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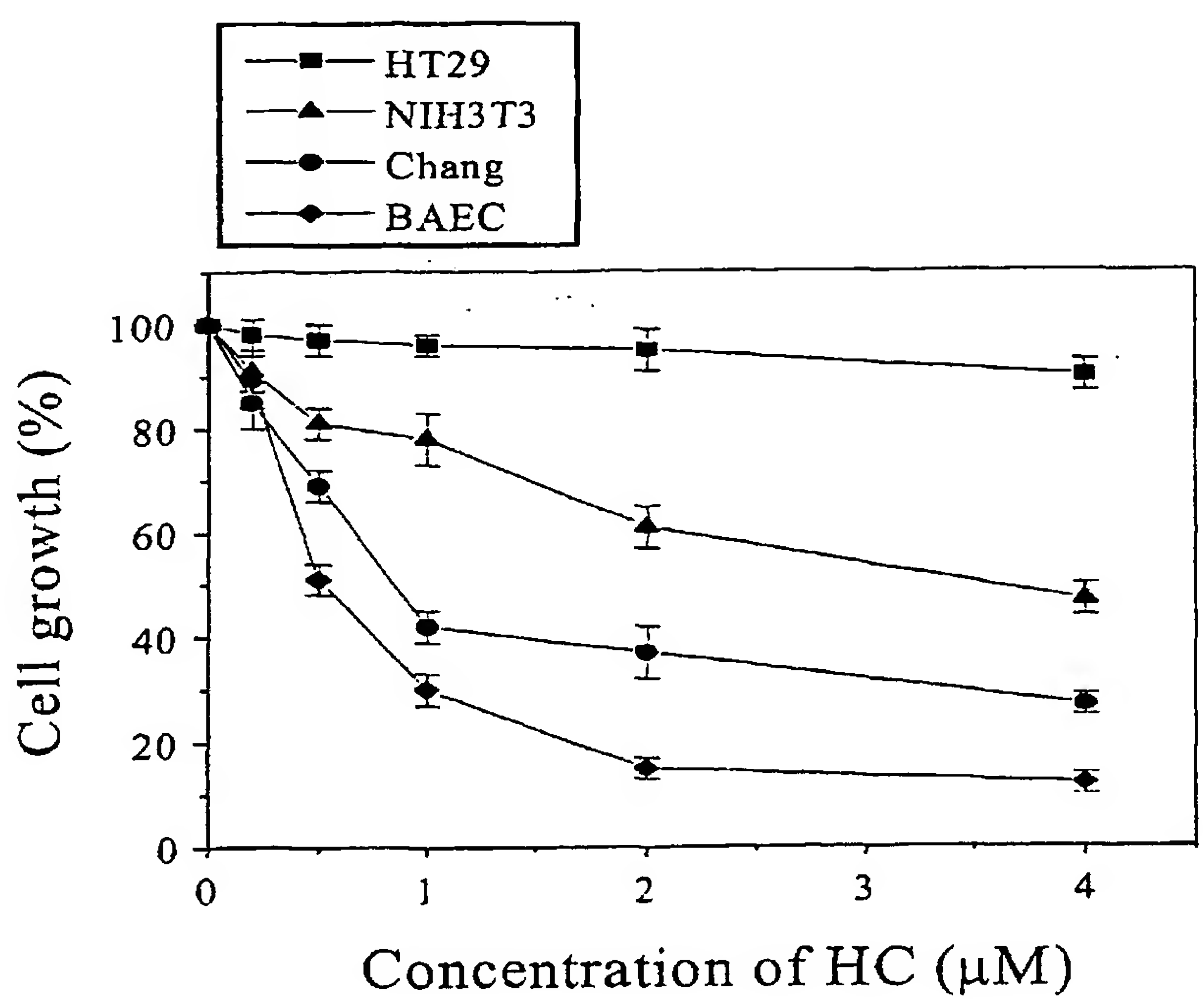
(54) Title: NOVEL CURCUMIN DERIVATIVES



(57) Abstract: The present invention relates to a novel curcumin derivative and a pharmaceutical composition, in particular to a novel curcumin derivative with anti-angiogenic activity and a pharmaceutical composition for treating or preventing a disease associated with unregulated angiogenesis.

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NOVEL CURCUMIN DERIVATIVES

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to a novel curcumin derivative and a pharmaceutical composition, in particular to a novel curcumin derivative with anti-angiogenic activity and a pharmaceutical composition for treating or preventing a disease associated with unregulated angiogenesis.

DESCRIPTION OF THE RELATED ART

Angiogenesis, the growth of new blood vessels, is essential for a number of physiological process such as embryonic development, wound healing, and tissue or organ regeneration. However, persistent unregulated angiogenesis drives angiogenic diseases such as rheumatoid arthritis, diabetic retinopathy, cancer, hemangioma and psoriasis (Andre, T., et al., 1998. *Rev. Med. Interne*. 19:904-9134; Battegay, E. J. 1995. *J. Mol. Med*. 73: 333-346; Carmeliet, P. and R. K. Jain. 2000. *Nature* 407:249-257; and Fidler, I. J. 2000. *Cancer J. Sci. Am*. 2:134-141).

The process is consisted of multi-steps such as stimulation of endothelial growth by tumor cytokines, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), degradation of extracellular matrix proteins by metalloproteinases,

migration of endothelial cells mediated by cell membrane adhesion molecules, endothelial cell proliferation and tube formation (Bussolino, F. et al., 1997. *Trends Biochem. Sci*. 22:251-256; Kuwano, M. et al., 2001. *Intern. Med.* 40:565-572; and Risau, W. 1994. *Arzneimittelforschung* 44:416-417). Therefore, inhibition of these processes is emerging as a promising new strategy for the treatment of cancer and other human diseases related with angiogenesis.

A new diverse class of angiogenesis inhibitors has been developed for this purpose. The inhibitors, which are natural or synthetic, include protease inhibitors, tyrosine kinase inhibitors, chemokines, interleukins, and proteolytic fragments of matrix proteins (Abedi, H. and I. Zachary. 1997. *J. Biol. Chem.* 272: 15442-15451; Cao, Y. 2001. *Int. J. Biochem. Cell Biol*. 33: 357-369; Fong, T. A et al., 1999. *Cancer Res.* 59:99-106; and Kwon, H. J. et al., 2001. *Acalycigorgia inermis. J. Microbiol. Biotechnol.* 11:656-662). These antiangiogenic molecules function in multiple ways, including the inhibition of endothelial cell proliferation, migration, protease activity, and tubule formation, as well as the induction of apoptosis (Folkman, J. and D. Ingber. 1992. *Semin. Cancer Biol.* 3:89-96; Kishi, K. et al., 2000. *Nippon Rinsho* 58:1747-1762; and Marme, D. 2001. *Onkologie* 1:1-5). The antiangiogenic function of many of these molecules is well documented *in vitro* and *in vivo*, and some are currently being tested in clinical trials (Deplanque, G. and A. L.

Harris. 2000. *Eur. J. Cancer* 36:1713-1724; Liekens, S. E. D. Clercq, and J. Neyts. 2001. *Biochem. Pharmacol.* 61:253-270; and Mross, K. 2000. *Drug Resist. Updat.* 3: 223-235).

Curcumin, a natural product found in the rhizome of *Curcuma longa*, is a potent chemopreventive agent that has been entered into the phase I clinical trials for chemoprevention by National Cancer Institute (Kelloff, G. J. et al., *J. Cell Biochem.* 1996, *26(suppl)*, 54). Curcumin showed a potent anti-carcinogenic activity against a broad range of tumor types including skin, forestomach, duodenal, and colon carcinogenesis (Rao, C. V. et al., *Cancer Res.* 1995, *55*, 259; Huang, M. T. et al., *Carcinogenesis* 1995, *16*, 2493; Huang, M. T. et al., *Cancer Res.* 1994, *54*, 5841; and Conney, et al., *Adv. Enzyme Regul.* 1991, *31*, 385). It has been postulated that the broad spectrum of anti-carcinogenic activity of curcumin may be due in part to angiogenesis inhibition (Mohan, R. et al., *J. Biol. Chem.* 2000, *275*, 10405; and Thaloor, D. et al., *Cell Growth & Differ.* 1998, *9*, 305). Curcumin and some of its derivatives including demethoxycurcumin (DC) and tetrahydrocurcumin (THC) were known as potent inhibitors of angiogenesis (Arbiser, J. L. et al., *Mol. Med.* 1998, *4*, 376). DC as well as THC, a urine metabolite of curcumin, showed a significant inhibition of corneal neovascularization, but the potential of the activity was relatively weaker than that of curcumin.

Throughout this application, various publications are referenced and citations are provided in parentheses. The disclosure of these publications in their entities are hereby incorporated by references into this application in order to more fully describe this invention and the state of the art to which this invention pertains.

SUMMARY OF THE INVENTION

The present inventors have made extensive investigation on novel curcumin derivative and as a result, a variety of derivatives with antiangiogenic activity have been synthesized.

Accordingly, it is an object of this invention to provide some curcumin derivatives.

It is another object of this invention to provide a pharmaceutical composition for treating or preventing a disease associated with unregulated angiogenesis.

It is still another object of this invention to provide a method for extracting from *Curcuma aromatica* curcumin and derivatives thereof.

Other objects and advantages of the present invention will become apparent from the detailed description to follow taken in conjugation with the appended claims and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 summaries the procedures to purify DC (demethoxycurcumin) from *Curcuma aromatica* in an improved yield compared to a conventional process.

Fig. 2 shows the cytotoxicity of DC or HC (hydrazinocurcumin). Panel A represents the effect of DC on viability of HUVECs. 24-well culture plate was coated with gelatin (2%) and incubated at 37℃ for 1h. DC treated in a dose-dependent manner and incubated for 72 h. The cells were then determined by trypan blue assay. Data represents the means ± SE of two different experiments. Panel B shows the results of trypan blue staining to evaluate *in vitro* toxicity of HC on tube-formed endothelial cells. Saturosporin, a cytotoxic agent, was used as an indicator of *in vitro* toxicity.

Fig. 3 demonstrates an inhibition of capillary tube formation by DC or HC. In panel A, HUVECs were seeded on the Matrigel coated wells at a density of 1×10^5 cells/well with or without bFGF (basic fibroblast growth factor). HUVECs were stimulated with bFGF and 5 μM DC was treated. Photographs were taken at 18 h after the drug treatment. Each sample was assayed in duplicated.

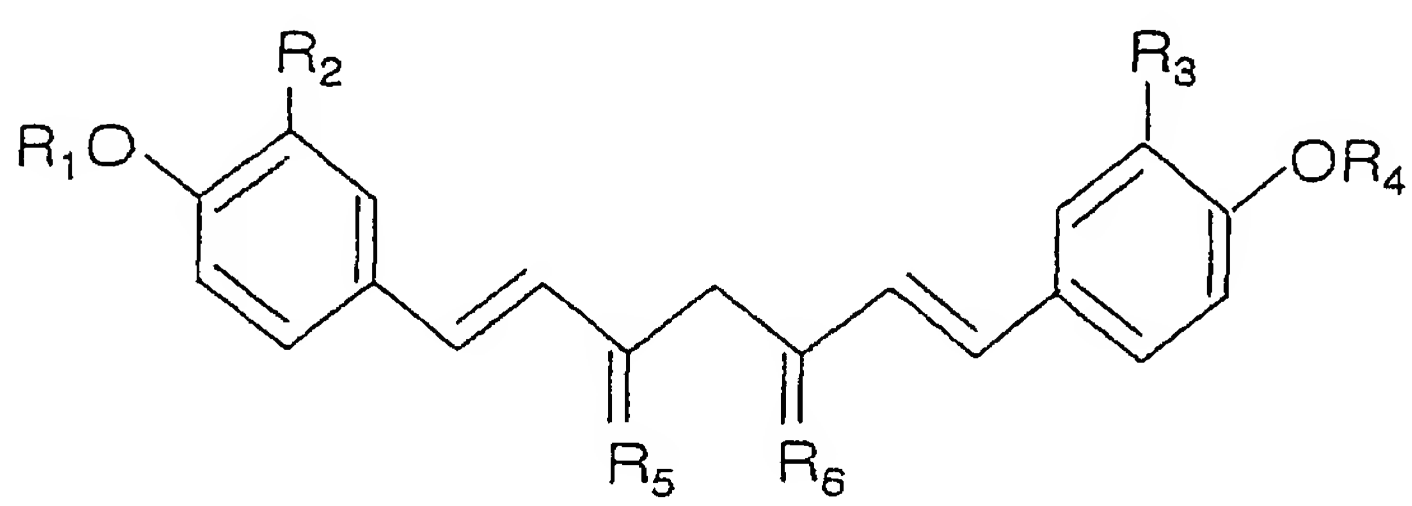
Fig. 4 shows effect of HC on the growth of various cell lines. Cell growth was measured using MTT colorimetric assay. Data represent mean ± SE from three independent experiments.

Fig. 5 shows the inhibitory effect of HC on endothelial cell invasion. Serum-starved BAECs left in serum-free

medium (Control) or treated with bFGF in the presence or absence of HC were used for invasion assay. A, Data represent mean ± S.E. from three independent experiments. B, Microscopic observation of invaded cells (x 100 magnification).

DETAILED DESCRIPTION OF THIS INVENTION

In one aspect of this invention, there is provided a curcumin derivative represented by the following formula I:



(I)

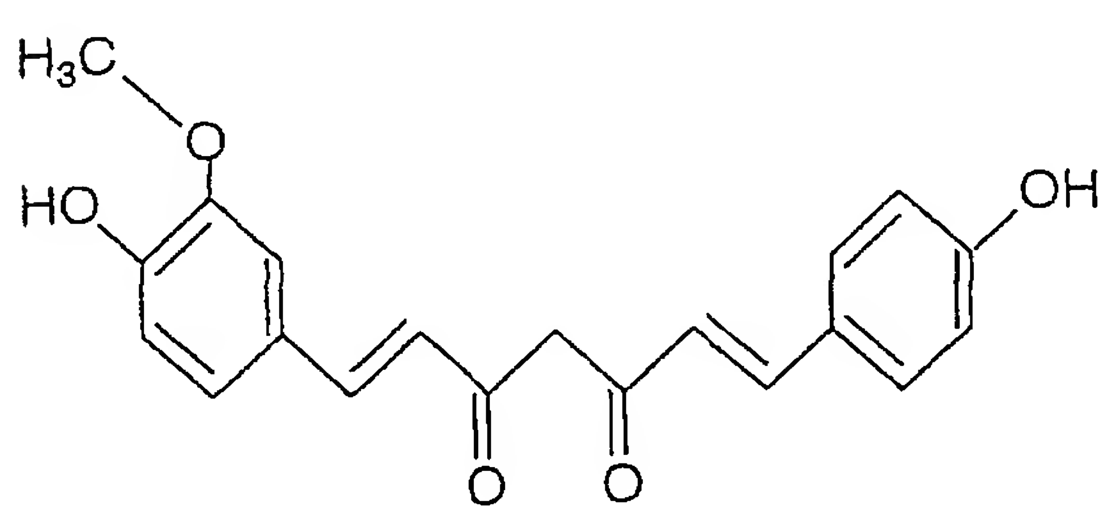
wherein R_1 represents H or lower alkyl group of 1-4 carbon atoms, R_2 represents H or lower alkoxy group of 1-4 carbon atoms, R_3 represents H or lower alkoxy group of 1-4 carbon atoms, R_4 represents H or lower alkyl group of 1-4 carbon atoms and both of R_5 and R_6 represent nitrogen or oxygen atoms; in which when both of R_5 and R_6 are nitrogen atoms, each of R_5 and R_6 is substituted with $-OR_7$ and R_7 is H, alkyl, cycloalkyl, aryl, alkaryl or aralkyl, or R_5 and R_6 form a ring structure with a hydrazine group and R_5 and R_6 are unsubstituted or independently substituted with alkyl, cycloalkyl, aryl, alkaryl or aralkyl; and in which when R_1 is H, R_2 is not methoxy, R_3 is not H or methoxy

group, R_4 is not H and both of R_5 and R_6 are not oxygen.

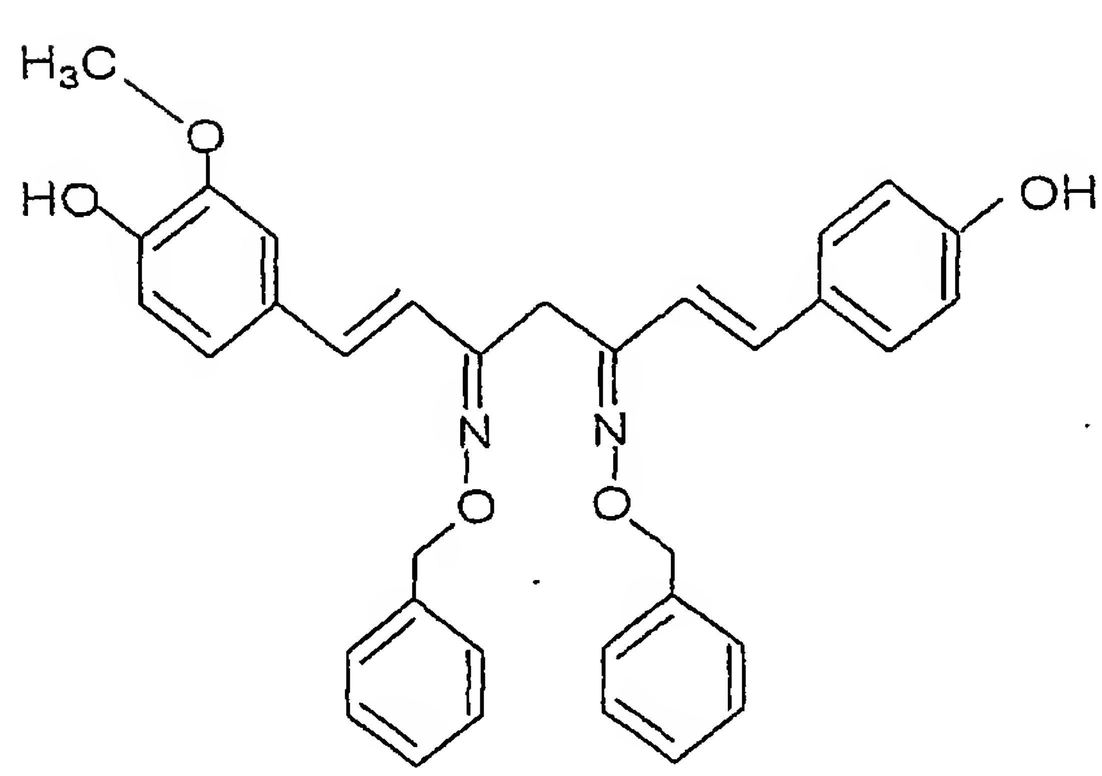
The present inventors have made efforts on synthesizing novel curcumin derivatives with examining activity against angiogenesis, cell specificity, toxicity and the like which are considerable factors in selecting a leading compound for drug.

The present derivatives are methylated, oxime and hydrazine derivatives of curcumin.

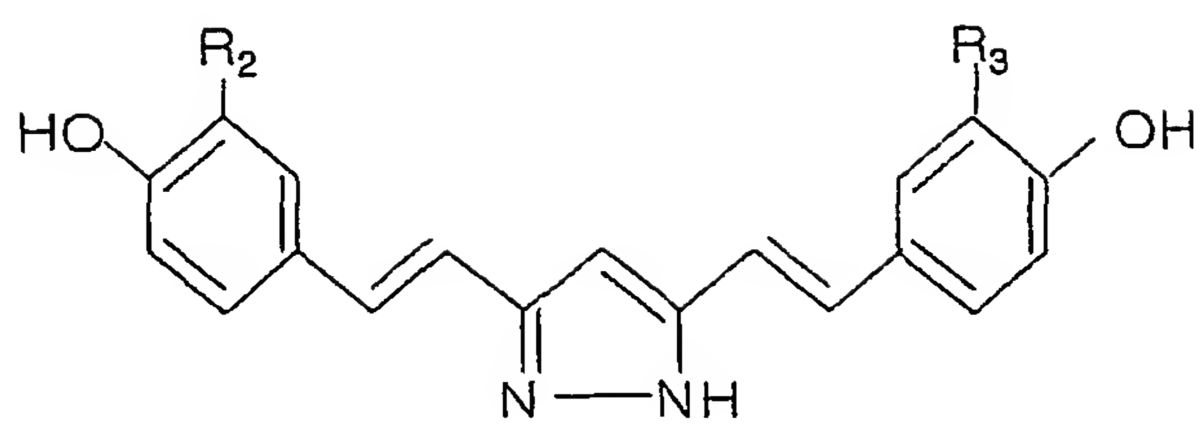
According to a preferred embodiment, the present derivative is represented by any one of the following formulae II, III, IV, V and VI:



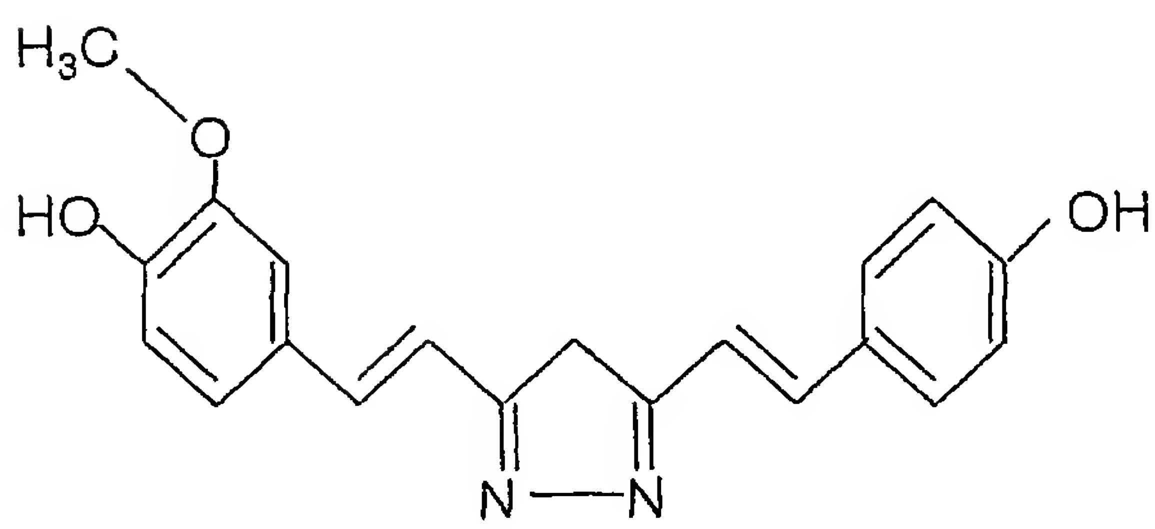
(II)



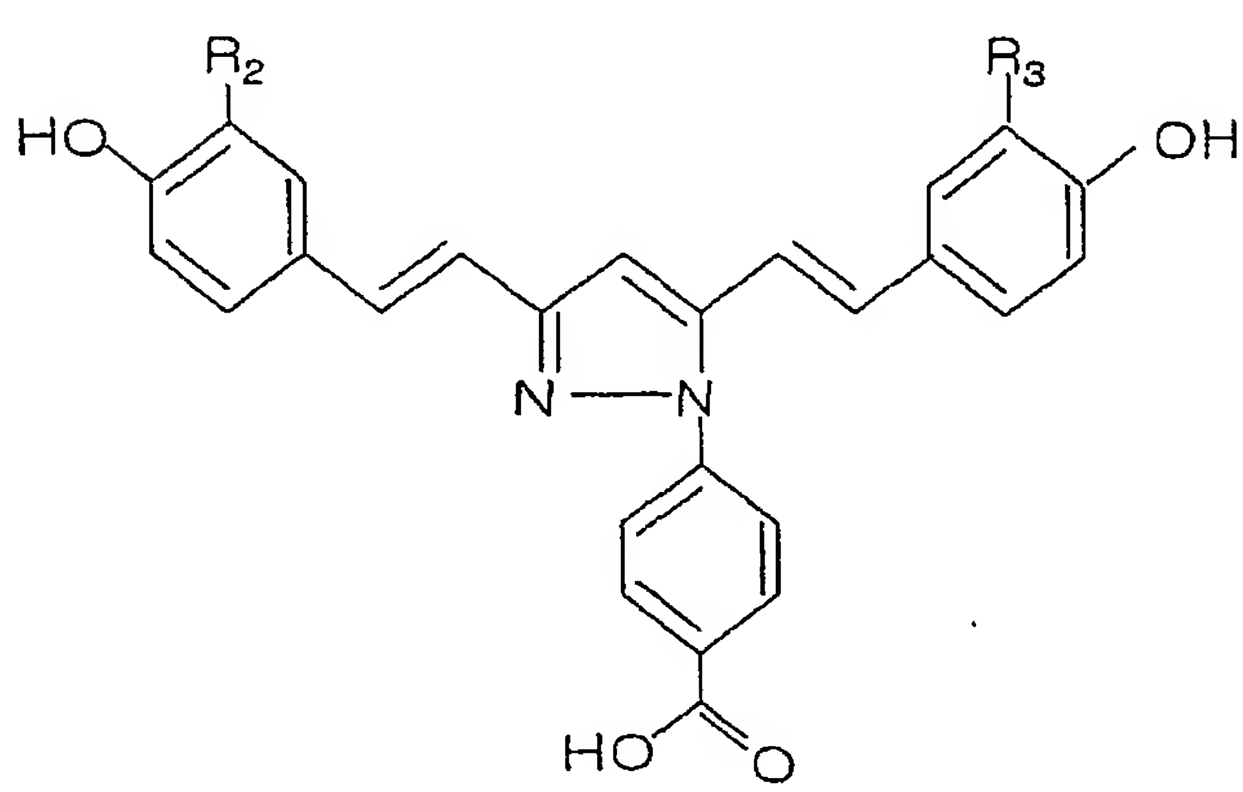
(III)



(IV)



(V)



(VI)

wherein R_2 and R_3 are the same as those in formula I.

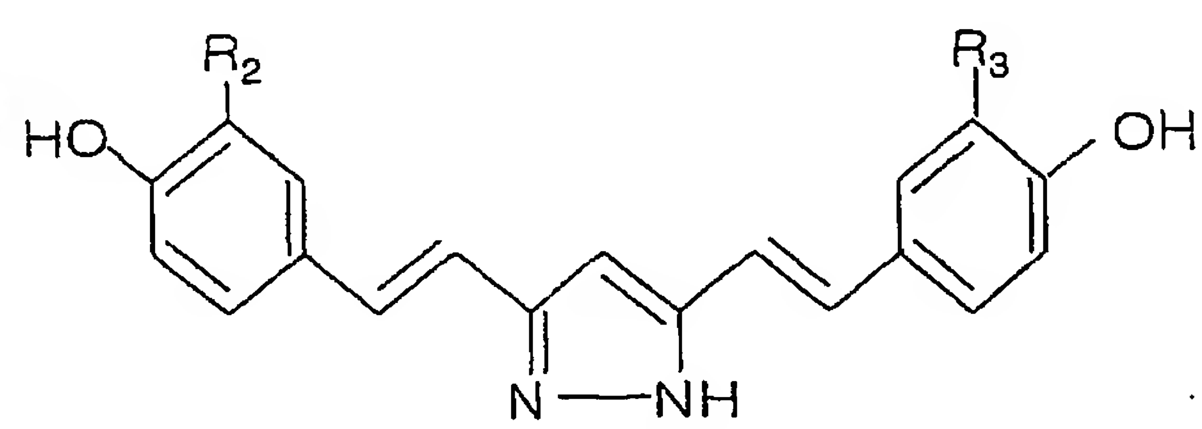
More preferably, R_2 and R_3 in formulae I, IV and VI independently represent H or methoxy group.

In another aspect of this invention, there is provided a pharmaceutical composition for treating or preventing a disease associated with unregulated angiogenesis, which

comprises: (a) a pharmaceutically effective amount of the curcumin derivative described above; and (b) a pharmaceutically acceptable carrier.

According to a preferred embodiment, the disease associated with unregulated angiogenesis, which may be treated or prevented with the present composition, is rheumatoid arthritis, diabetic retinopathy, cancer, hemangioma or psoriasis. More preferably, the disease associated with unregulated angiogenesis is cancer.

According to a preferred embodiment, the curcumin derivative in the present composition is a hydrazinocurcumin. It is more preferred that the hydrazinocurcumin is represented by the following formula IV:



(IV)

wherein R_2 and R_3 are the same as those in formula I.

In the present hydrazinocurcumin of the formula IV, it is more advantageous that R_2 and R_3 independently represent H or methoxy group. Most preferably, in formula IV, both of R_2 and R_3 are methoxy group.

In the pharmaceutical compositions of this invention, the pharmaceutically acceptable carrier may be conventional one for formulation, including lactose,

dextrose, sucrose, sorbitol, mannitol, starch, gum acacia, calcium phosphate, alginate, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrup, methyl cellulose, methylhydroxy benzoate, propylhydroxy benzoate, talc, stearic acid, magnesium and mineral oil, but not limited to. The pharmaceutical compositions of this invention, further may contain wetting agent, sweetening agent, emulsifying agent, suspending agent, preservatives, flavors, perfumes, lubricating agent, or mixtures of these substances. The pharmaceutical composition of this invention may be administered orally or parenterally.

The correct dosage of the pharmaceutical compositions of this invention will vary according to the particular formulation, the mode of application, age, body weight and sex of the patient, diet, time of administration, condition of the patient, drug combinations, reaction sensitivities and severity of the disease. It is understood that the ordinary skilled physician will readily be able to determine and prescribe a correct dosage of this pharmaceutical compositions. An exemplary daily dosage unit for human host comprises an amount of from about 0.001 mg/kg to about 100 mg/kg.

According to the conventional techniques known to those skilled in the art, the pharmaceutical compositions of this invention can be formulated with pharmaceutical acceptable carrier and/or vehicle as described above,

finally providing several forms including a unit dosage form. Non-limiting examples of the formulations include, but not limited to, a solution, a suspension or an emulsion, an extract, an elixir, a powder, a granule, a tablet, a capsule, a liniment, a lotion and an ointment.

In still another aspect of this invention, there is provided a method for extracting from *Curcuma aromatica* curcumin and derivatives thereof, which comprises contacting *Curcuma aromatica* with 70-98% methanol solution for 2-5 hours under heat treatment at 55-65℃.

Generally, an extraction from natural source may be carried out with a suitable organic solvent such as lower alkyl alcohol, chloroform, dichloromethanol and lower alkyl polyol. According to the present method, it is the most preferable that the extraction is performed with methanol. According to the most preferred embodiment, the extraction comprises contacting *Curcuma aromatica* with about 95% methanol solution for about 3 hours under heat treatment at about 60℃. Such conditions for extraction are optimal in terms of the final yield of physiologically active ingredient.

Through the extraction method, curcumin and its derivative such as demetoxycurcumin and bisdemetoxycurcumin can be obtained in a mixture.

The following specific examples are intended to be

illustrative of the invention and should not be construed as limiting the scope of the invention as defined by appended claims.

Example I: Extraction and Purification of Demethoxycurcumin (DC) from *Curcuma aromatica*

I-1: Methods for Extraction of DC from *Curcuma aromatica*

I-1-a: Analysis of DC Contents Depending on Extracting Solvents

In an effort for high yield of DC from Curcuma, the extracted content of DC was measured in 20 g Curcuma depending on each extracting solvents. The DC content extracted was measured by HPLC after maceration of Curcuma in methanol (85%), ethanol (85%) or methanol:dichloromethanol (1:1) for 5 days (*Planta Medica*. 66:396-398(2000)). According to the results, methanol (85%) was chosen as the preferred extracting solvent among them.

Table 1. DC contents depending on extracting solvents

Components of Curcuma	Methanol (85%)	Ethanol (85%)	Methanol:Dichloromethanol (1:1)
Curcumin (CUR)	235 mg	176 mg	59 mg
Demethoxycurcumin (DC)	118 mg	106 mg	47 mg
Bisdemethoxycurcumin (BDC)	147 mg	129 mg	105 mg

I-1-b: Analysis of DC Contents Depending on Heating

The extracted DC content was measured in each extracting solvents for 2-5 h depending on heating at 55-

65℃. Among the above experiments, heating with 85% methanol showed the highest amount of DC extraction. The result without heat treatment showed 118 mg and additional heating led to 347 mg of DC content under the extraction by 85% methanol. The high extraction efficiency of heating was applied in use of ethanol as well.

Table 2. DC contents depending on heating

w/o heating			
Components of Curcuma	Methanol (85%)	Ethanol (85%)	Methanol:Dichloromethanol(1:1)
CUR	235 mg	176 mg	59 mg
DC	118 mg	106 mg	47 mg
BDC	147 mg	129 mg	105 mg
w/ heating (2-5 h, 55-65℃)			
Components of Curcuma	Methanol (85%)	Ethanol (85%)	Methanol:Dichloromethanol(1:1)
CUR	753 mg	588 mg	71 mg
DC	347 mg	235 mg	47 mg
BDC	459 mg	353 mg	106 mg

I-1-c: Analysis of DC Contents Depending on Methanol Ratio

The extracted DC contents measured by HPLC depending on methanol ratio from 100% to 70% showed that 95% methanol is preferred methanol ratio to yield the highest DC extraction.

According to the results, about 1.5-fold higher yield

of DC extraction can be acquired by heating at 55-65℃ for 2-5 h in 70-98% methanol comparing to conventional maceration for 5 days.

Furthermore, the optimum condition for DC extraction from Curcuma was found to be the extraction by use of 95% methanol with heating (60℃) for 3 h.

Table 3. DC contents depending on methanol ratio

Components of Curcuma	Methanol ratio					
	100%	95%	90%	85%	80%	70%
CUR	764 mg	765 mg	735 mg	753 mg	712 mg	694 mg
DC	294 mg	359 mg	353 mg	347 mg	341 mg	335 mg
BDC	353 mg	471 mg	459 mg	459 mg	459 mg	459 mg

Example II: Isolation and Purification of Physiologically Active Materials

DC was prepared from *Curcuma aromatica*. Briefly, the rhizome of turmeric was extracted with 95% methanol for 3 h at 60℃ and concentrated *in vacuo*. Ethylacetate extract was filtered and separated by silica gel (70-230 mesh) column chromatography (ψ 6x17 cm), with a solvent system of hexane/chloroform/methanol (3:9:1). Among the fractions obtained, physiologically active fraction was further separated with silica gel (70-230 mesh) column chromatography (ψ 3x15 cm), with the same solvent system. Active fraction was further purified with preparative thin layer chromatography (silica gel 60 F_{254}) using

hexane/chloroform/methanol (3:9:1). Final purification of active compound was achieved by HPLC (SHIMAZU, 0.1% trifluoroacetic acid: acetonitrile = 40:60, flow rate: 1 mℓ /min). The chemical structure of and molecular weight of DC were analyzed through Mass and ^{1}H-NMR (500 MHz), ^{13}C-NMR (250 MHz) spectrophotometries, respectively. The above procedures are summarized in Fig. 1.

Example Ⅲ: Dimethylation of DC

5 mg DC (14.7 μmol) was dissolved in 0.5 mℓ acetone, added 18.3 μℓ methyl iodide (294 μmol) and 20.3 mg K_2CO_3 (147 μmol) and reacted for 12 h with stirring. 2 mg of dimethyl DC was separated by TLC and HPLC analysis.

The molecular weight and formula of the dimethyl DC were identified as below using ESI-MS and ^{1}H-NMR: M.W. 365; Formula $C_{22}H_{21}O_5$; maximum absorbance 200 nm, 430 nm; and yellowish color

Example Ⅳ: Oxime-Derivative of DC

10 mg DC (29.4 μmol) was dissolved in 1 mℓ methanol, added 10.81 mg benzyl hydroxyl amine (67.75 μmol) and 9.4 μℓ triethyl amine (67.75 μmol), and reacted for 12 h with heat treatment under stirring. 3 mg oxime-derivative of DC was separated by TLC and HPLC analysis.

The molecular weight and chemical formula of the oxime-derivative of DC were identified as below using ESI-MS and ^{1}H-NMR: M.W. 547; Formula $C_{34}H_{31}O_5N_2$; maximum

absorbance 200 nm, 325 nm and light yellowish color

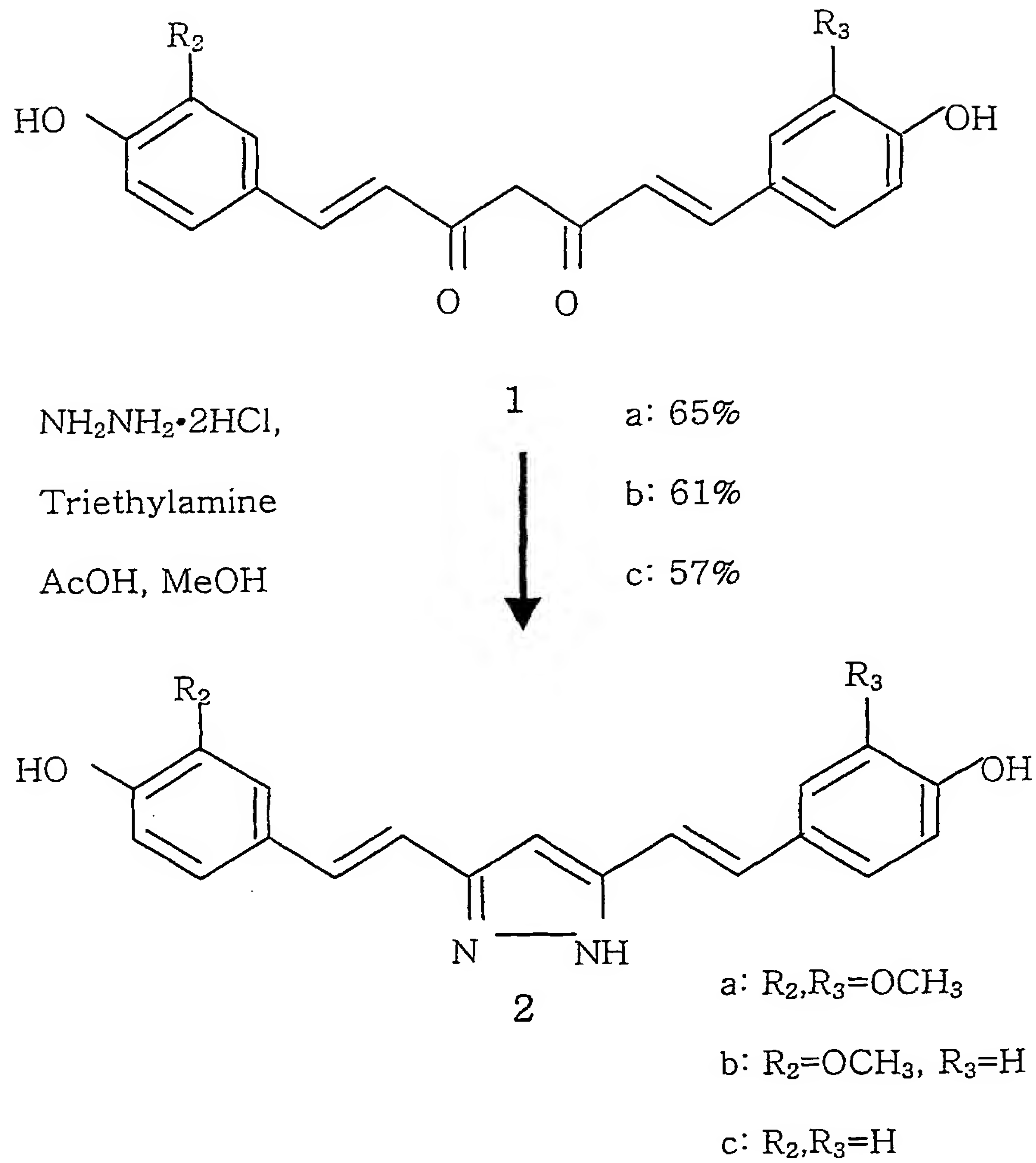
Example V: Hydrazine-Derivatives of DC

V-1: General Procedures

Curcumins was obtained by Sigma (St. Louis, MO). Chromatography purification: Silica gel column chromatography (Merck, Darmstadt, Germany), thin-layer chromatography (TLC, Merck), and high performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) were used to perform general purification procedures. Spectral analysis: The HRFAB-MS spectra were obtained using a Jeol JMS-HX 110 mass spectrometer. The NMR spectra were recorded in $CDCl_3$ solutions on a Varian Unity 500 spectrometer. The proton and carbon NMR spectra were measured at 500 and 125 MHz, respectively. All chemical shifts were recorded according to an internal Me_4Si. All solvents used were of spectral grade or distilled from glass prior to use.

The general procedures for synthesis of derivatives are shown in Schemes 1 and 2:

Scheme 1



V-2: <u>Synthesis of Hydrazinocurcumin (2a)</u>

Purified 1a (R_1, R_2 = OCH_3; 10 mg, 27 μmol) was dissolved in methanol (2 mℓ), and hydraziniumdihydrochloride (14 mg, 13.5 μmol) and triethylamine (18.8 *μℓ*, 13.5 μmol) were added to the solution. In the presence of catalytic amount of acetic acid, the reaction mixture was incubated for 24 h at room temperature with gentle stirring. The solvent was evaporated *in vacuo* and the residue purified by preparative TLC ($CHCl_3$:MeOH = 6:1; R*f* = 0.4) and HPLC

(semi-preparative C18 column; acetonitrile:H_2O = 50:50; flow rate = 3 mℓ/min; retention time: 21 min) gave 2a as pale yellow gum (6.19 mg, 65%) which analyzed by HRFAB-MS. Exact mass was calcd for $C_{21}H_{20}N_2O_4$: 364.1423, observed (M+H) 365.1501.

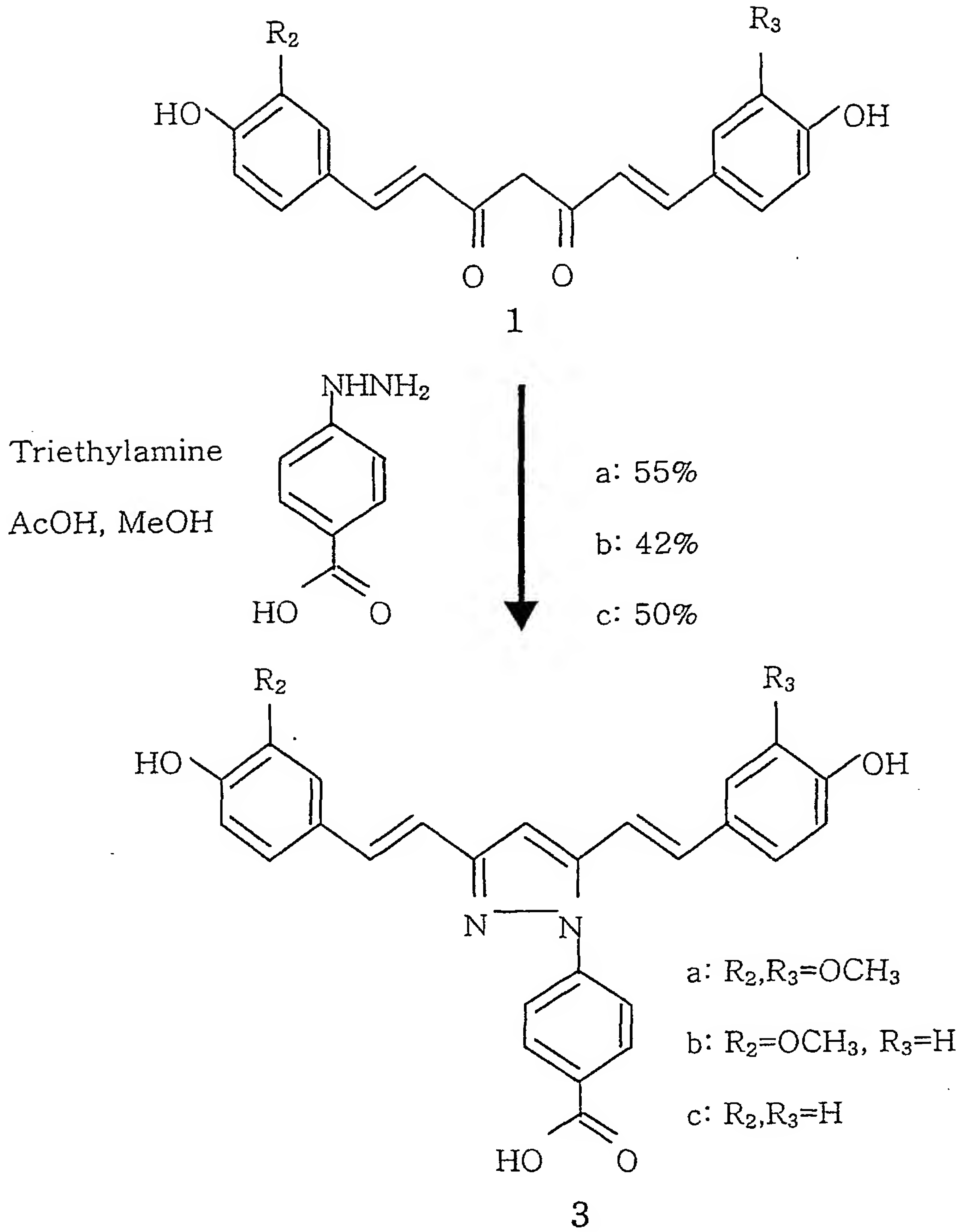
V-3: Synthesis of Hydrazinodemethoxycurcumin (2b)

Utilizing the same protocol as described for the synthesis of **2a**, **2b** (5.9 mg, 61%) was obtained from **1b** (10 mg, 29 μmol), hydraziniumdihydrochloride (14 mg, 13.5 μmol), and triethylamine (18.8 μℓ, 13.5 μmol). Preparative TLC ($CHCl_3$:MeOH = 6:1; *Rf* = 0.32) gave **2b** as a pale yellow gum which analyzed by HRFAB-MS. Exact mass calcd for $C_{20}H_{18}N_2O_3$: 334.1317, observed (M+H) 335.1393.

V-4: Synthesis of Hydrazinobisdemethoxycurcumin (2c)

Utilizing the same protocol as described for the synthesis of **2a**, **2c** (5.5 mg, 57%) was obtained from **1c** (10 mg, 32 μmol), hydraziniumdihydrochloride (14 mg, 13.5 μmol), and triethylamine (18.8 μℓ, 13.5 μmol). Preparative TLC ($CHCl_3$:MeOH = 6:1; *Rf* = 0.26) gave **2c** as a pale yellow gum which analyzed by HRFAB-MS. Exact mass calcd for $C_{19}H_{16}N_2O_2$: 304.1212, observed (M+H) 305.1291.

Scheme 2



V-5: <u>Synthesis of Hydrazinobenzoylcurcumin (3a)</u>

To a solution of **1a** (10 mg, 27 μmol) in methanol (2 mℓ) was added 4-hydrazinobenzoic acid (20 mg, 13.5 μmol), triethylamine (18.8 μℓ, 13.5 μmol), and catalytic amount of

acetic acid. After incubation for 24 h, the solvent was evaporated *in vacuo* and the residue purified by preparative TLC ($CHCl_3$:MeOH = 4:1; R*f*,=0.5) and HPLC (semipreparative C18 column; acetonitrile:H_2O = 50:50; flow rate = 3 mℓ/min; retention time: 15 min) gave **3a** as a dark orange powder (7.18 mg, 55%) which analyzed by HRFAB-MS. Exact mass calcd for $C_{28}H_{24}N_2O_6$: 484.1634, observed (M+H) 485.1715.

V-6: Synthesis of Hydrazinobenzoyldemethoxycurcumin (3b)

Utilizing the same protocol as described for the synthesis of **3a**, **3b** (5.52 mg, 42%) was obtained from **1b** (10 mg, 29 μmol), 4-hydrazinobenzoic acid (20 mg, 13.5 μmol), and triethylamine (18.8 μℓ, 13.5 μmol). Preparative TLC ($CHCl_3$:MeOH = 4:1; R*f*,=0.41) gave **3b** as a dark orange powder which analyzed by HRFAB-MS. Exact mass calcd for $C_{27}H_{22}N_2O_5$: 454.1529, observed (M+H) 455.1611.

V-7: Synthesis of Hydrazinobenzoylbisdemethoxycurcumin (3c)

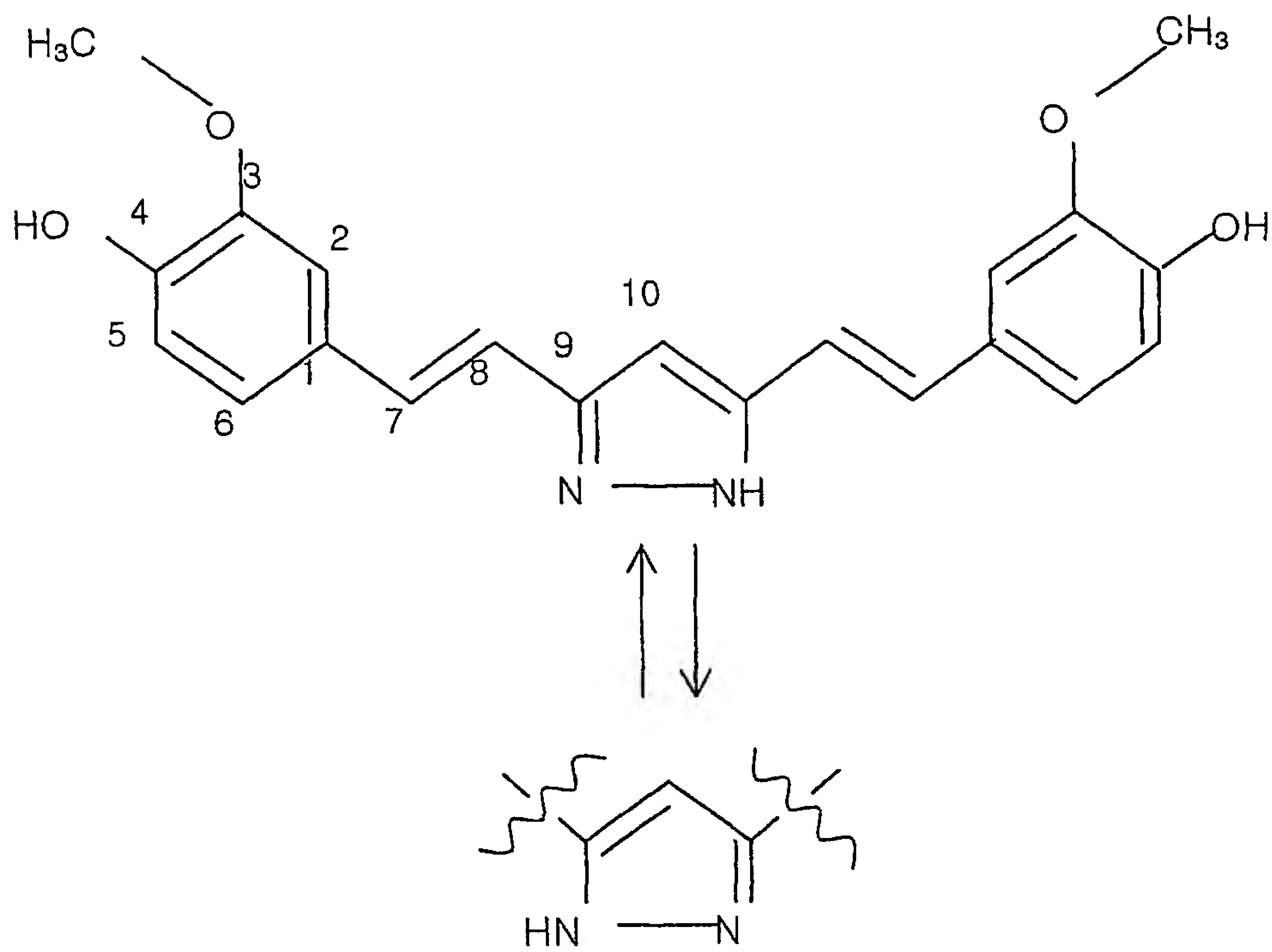
Utilizing the same protocol as described for the synthesis of **3a**, **3c** (6.78 mg, 50%) was obtained from **1c** (10 mg, 32 μmol), 4-hydrazinobenzoic acid (20 mg, 13.5 μmol), and triethylamine (18.8 μℓ, 13.5 μmol). Preparative TLC ($CHCl_3$:MeOH = 4:1; R*f*,=0.34) gave **3c** as a dark orange

powder which analyzed by HRFAB-MS. Exact mass calcd for $C_{26}H_{20}N_2O_4$: 424.1423, observed (M+H) 425.1501.

V-8: Structure Determination of Hydrazinocurcumin (HC)

HC (2a), a synthetic analog of curcumin was obtained as pale yellow gum which was analyzed for $C_{21}H_{20}N_2O_4$ by HRFAB-MS, thus 13 degree of unsaturation. The structure of this compound was determined by combined 2D NMR experiments as well as comparison of spectral data with those obtained for curcumin (Table 4). All of the signals of protons and carbons of the benzene ring were readily assigned on the basis of the results of ^1H COSY, gHSQC, and gHMBC experiments. However, considerable differences were observed for the NMR signals corresponding to the protons and carbons at the side chain. The ^{13}C NMR spectra showed a broad signal at δ 131.1 (d) which correlated to both of the protons at δ 7.09 and 6.94 in the gHSQC spectra. The ^1H COSY data revealed that these protons were coupled to each other with very large coupling constant (J = 16.1 Hz).

Table 4. Structure Analysis of HC



	^1H	^{13}C	HMBC	ROESY
1		129.5 s		
2	7.16, 2H, br s	110.0 d	3, 4, 6, 7	7, 8, OMe
3		148.8 s		
4		148.0 s		
5	6.78, 2H, d (7.8)	116.1 d	1, 3	6
6	6.97,2H,dd (7.8,1.5)	121.2 d	2, 4, 7	5, 7
7	7.09, 2H, d (16.1)	131.1 d	2, 6	2, 6, 10
8	6.94, 2H, d (16.1)	131.1 d	10	2, 10
9		N. A.[a]		
10	6.64, 1H, s	99.9 d		7, 8
OMe	3.86, 6H, s	56.2 d	3	2

[a] Signal was not observed

Therefore, these were thought to be the olefinic protons of a *trans*-double bond. This interpretation was supported by ROESY experiments in which cross peaks were observed between both of the olefinic protons and aromatic

ones at δ 7.16 and 6.97.

The NMR data of HC contained another signals of an additional methine group; δ_H 6.64, δ_C 99.9. Since the intensity of the proton signal was almost half of others, this methine must be corresponded to the C-10 symmetric center of the molecule. Although signal of the C-9 carbon was not observed due to the rapid tautomerization between two forms of a pyrazole moiety, the upfield shift of the C-10 methine carbon in the ^{13}C NMR data indicated the placement of an olefinic carbon bearing an electronegative atom at adjacent location. Data supporting this interpretation were provided by 2D NMR experiments in which the gHMBC-correlation between H-8 and C-10 as well as the ROESY cross peaks between H-8 and H-10 were observed. Thus, the structure of HC was defined as an analog of curcumin bearing a pyrazole moiety in the middle of chain.

Example VI: IC_{50} Values of the Curcumins and Synthetic Derivatives for BAECs

To evaluate anti-angiogenic activites of synthetic curcumin derivatives, each compounds was treated on endothelial cell proliferation and assayed using MTT colorimetric assay. Among the 6 derivatives tested, HC showed the most potent growth inhibitory activity against BAECs with an IC_{50} of 0.52 μM (Table 5). The inhibitory potency of HC against BAECs proliferation was over 30-fold

higher than that of curcumin. Hydrazine derivatives with bulky benzoic acid also showed an enhanced anti-proliferative activity against BAECs. However, the potency of the derivatives was relatively weaker than that of HC.

Table 5. IC_{50} values of curcumins and synthetic derivatives for BAECs.

	R_1	R_2	BAECs, IC_{50} (M)[a]
Curcumins, 1	OCH_3	OCH_3	$1.5 \pm 0.3 \times 10^{-5}$
	OCH_3	H	$2.2 \pm 0.5 \times 10^{-5}$
	H	H	$5.3 \pm 0.6 \times 10^{-5}$
Hydrazino curcumins, 2	OCH_3	OCH_3	$5.2 \pm 0.4 \times 10^{-7}$
	OCH_3	H	$1.8 \pm 0.3 \times 10^{-6}$
	H	H	$5.8 \pm 0.2 \times 10^{-6}$
Hydrazinobenzoyl curcumins, 3	OCH_3	OCH_3	$9.3 \pm 0.4 \times 10^{-7}$
	OCH_3	H	$2.4 \pm 0.1 \times 10^{-6}$
	H	H	$8.7 \pm 0.2 \times 10^{-6}$

[a] IC_{50} values are the mean ±SE from three independent experiments.

Example VII: Cytotoxicity of DC or HC on Blood Vessel Endothelial Cells

In an effort to evaluate toxicity of the above isolated DC or HC on blood vessel endothelial cells, growth inhibition assay was performed with the DC or HC.

5×10^3 HUVECs/well of 96-well plate were cultured with 1 mℓ M199 supplemented with 20% serum at 37℃ in a 5% CO_2 incubator for 3 h. About 80% growth of HUVECs on each well was rinsed with 1mℓ of PBS, supplemented with new 20% serum-M199 medium and treated with DC in a concentration of 2.5-25 μg/mℓ. After 72 h, MTT reagent and DMSO were added, and then growth rate was calculated

with ELISA(OD_{540}) results compared to control.

As shown in Fig 2A, the purified DC as the present invention showed negligible cellular toxicity at 10 μg/mℓ treatment and resulted around 10% cellular toxicity at 20 μg/mℓ treatment on HUVECs.

In the case of HC, which is represented by 2a in Scheme 1, the toxicity of HC (300 nM) on BAECs was analyzed compared to staurosporin (10 μM) for the same duration of anti-angiogenic assay. While staurosporin resulted in severe cell death, HC did not show any cellular toxicity (Fig. 2B).

According to the above results, anti-angiogenic treatment was performed at 5 μg/mℓ treatment of DC and 100-300 nM of HC in which a sufficient anti-angiogenic effect was gained without any cellular toxicity.

Example Ⅷ: Inhibition of Capillary Tube Formation by DC or HC

Matrigel (250 μℓ, 10 mg/mℓ) was placed in a 24-well culture plates and polymerized for 30 min at 37℃. The BAECs (1×10^5 cells) were seeded on the surface of the Matrigel and treated with bFGF (30 ng/mℓ). Then, HC, which is represented by 2a in Scheme 1, was added and incubation was continued for 6-18 h. The morphological changes in the cells and tubes formed were observed under a microscope and photographed at ×100 magnification using JVC digital camera (Victor, Yokohama, Japan).

In an effort to confirm the inhibitory role of DC on capillary tube formation, 1 X 10^4 HUVECs/well of 24-well plate were cultured in 1 mℓ media containing 5 μg/mℓ DC at 37℃ in a 5% CO_2 incubator. In the course of incubation, the formation of capillary tube was checked by taking pictures at a 40X magnification using ImagePro Plus software (Media Cybernetics, Inc.) in a time dependent manner (Fig. 3A).

The inhibitory effect of HC on capillary tube formation was evaluated using BAECs in same procedures of the above assay for DC (Fig. 3B).

As shown in Fig. 3, DC and HC effectively inhibited the capillary tube formation induced by bFGF. However, the inhibitory effect of HC was found to be more potent than that of DC.

Example Ⅸ: Growth Inhibitory Effect of HC on Endothelial Cells

Early passages (5-7 passages) of bovine aortic endothelial cells (BAECs) were kindly provided by Dr. Jo at the NIH of Korea. BAECs were grown in MEM supplemented with 10% fetal bovine serum. HT29 (colon carcinoma), NIH3T3 (mouse normal fibroblast), and Chang (normal liver) cells were maintained in RPMI1640 containing 10% fetal bovine serum. All cells were grown at 37°C in a humidified atmosphere of 5% CO_2. Cell growth assay was carried out using MTT colorimetric assay.

Exponentially growing cells were seeded at a density of 5×10^3 cells/well in a 96-well plate. The cells were incubated in growth media for 24 h. Various concentrations of curcumin derivatives were added to each well and incubated for up to 72 h. After 72 h, 50 $\mu\ell$ of MTT (2 mg/mℓ stock solution, Sigma) was added and the plate was incubated for an additional 4 h. After removal of the culture supernatants, 150 $\mu\ell$ of DMSO was added. The plate was read at 540nm using a microplate reader (Bio-Tek Instruments, Inc., Winooski, Vermont).

Anti-proliferative activity of HC, which is represented by 2a in Scheme 1, was investigated to determine the cell line specificity. Several epithelial and fibroblast cells including, HT29, colon carcinoma, NIH3T3, normal fibrolbast, Chang, normal liver cells, and BAECs, were examined and the result showed that HC inhibited the proliferation of each cell lines with a different activity spectrum (Fig. 4). Interestingly, there was high endothelial cell specificity on the anti-proliferative activity of HC. Specificity factors over 20 were obtained versus HT29, colorectal carcinoma cells. Other normal cell lines also showed a degree of different sensitivity to HC with BAECs. In contrast to HC, curcumin, the parental compound, inhibited the proliferation of these cells in relatively non-selective manner (data not shown). These data suggest that HC inhibits cellular proliferation with an endothelial cell

specificity.

Example X: Inhibitory Effect of HC on Endothelial Cell Invasion

The invasiveness of the endothelial cells was performed *in vitro* using a Transwell chamber system with polycarbonate filter inserts. The lower side of the filter was coated with 10 $\mu\ell$ of gelatin (1 mg/mℓ), whereas the upper side was coated with 10 $\mu\ell$ of Matrigel (3 mg/mℓ). Exponentially growing cells (1×10^5 cells) were placed in the upper part of the filter and HC was applied to the lower part for 30 min at room temperature before seeding. The chamber was then incubated at 37°C for 18 h. The cells were fixed with methanol and stained with hematoxylin/eosin. The cell invasion was determined by counting whole cells on the lower side of the filter using an optical microscope at × 100 magnification.

Endothelial cell invasion and the formation of tubular structure as well as cellular proliferation are essential steps for angiogenic process. Thus, the effect of HC on BAECs invasion was evaluated. bFGF was used as a chemoattractant. HC, which is represented by 2a in Scheme 1, potently inhibited bFGF-induced BAECs invasion at nanomolar concentration (Fig. 5).

Example XI: Inhibitory Effect of DC, HC and their derivatives on Chorioallantoic Angiogenesis

The above *in vitro* results indicating the anti-angiogenic effect of DC, HC and their derivatives were further confirmed by *in vivo* chorioallantoic membrane (CAM) assay as below.

Fertilized chick eggs were kept in a humidified incubator at 37℃ for 3 days. About 2 mℓ of egg albumin was then removed with a hypodermic needle allowing the CAM and yolk sac to drop away from the shell membrane. On day 3.5, the shell was punched out and removed and the shell membrane peeled away. At the stage of a 4.5-day old chick embryo, a DC, HC (represented by 2a in Scheme 1) or their derivatives (20 μg/egg)-loaded thermanox coverslip was air-dried and applied to the CAM surface. Two days later, 2 mℓ of 10% fat emulsion was injected into the chorioallantois and the CAM was observed under a microscope. Since retinoic acid (RA) is known as an anti-angiogenic compound, 20 μg/egg RA was used as a positive control for anti-angiogenic responses. When the CAM treated with a sample showing an avascular zone to a similar degree of RA-treated CAM that had little vessels compared to empty coverslip, the response was scored as positive, and calculated based on the percentage of positive eggs to the total number of eggs tested.

Independent experiment was repeated three times and at least more than 20 eggs were examined (Table 6).

Table 6. Inhibition of angiogenesis of CAM *In vivo*.

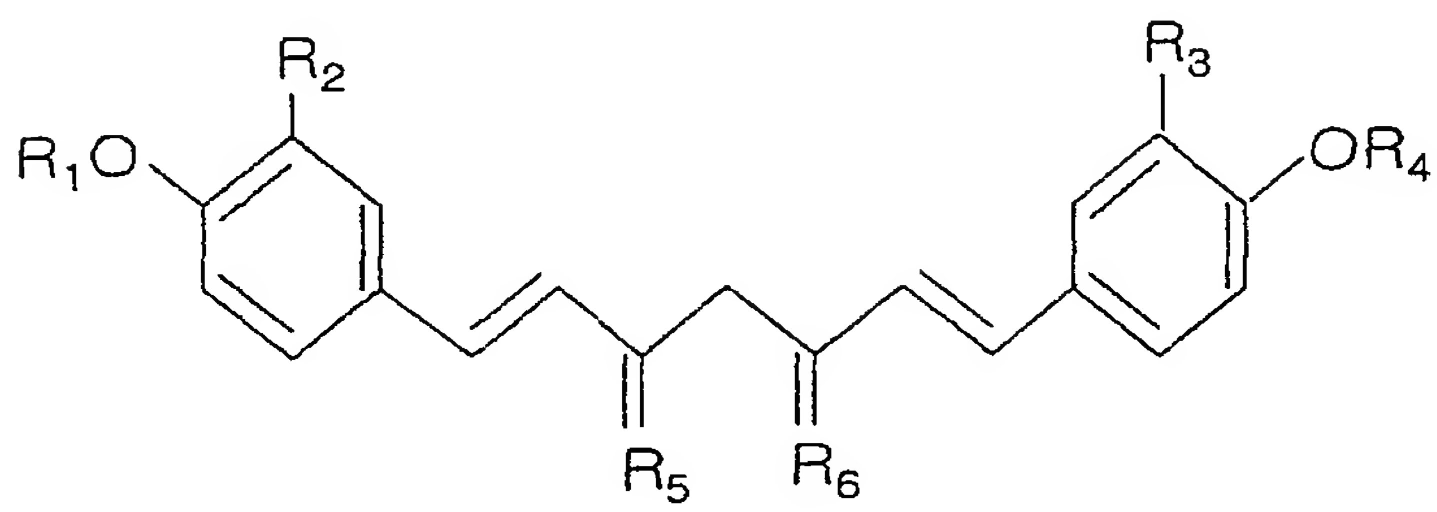
	Amount/disc	Inhibition (%)
Control	Methanol (2$\mu\ell$)	15
Retinoic acid	20 μg	89
DC	20 μg	86
Dimethyl DC	20 μg	85
Oxime derivative	20 μg	80
HC (2a)	20 μg	94

The equivalent inhibitory effect of DC and more potent inhibitory effect of HC than that of Retinoic acid, a well-known anti-angiogenic agent, shown in Table 6 indicate that the DC, HC and its derivatives of the present invention are also anti-angiogenic agents.

Having described a preferred embodiment of the present invention, it is to be understood that variants and modifications thereof falling within the spirit of the invention may become apparent to those skilled in this art, and the scope of this invention is to be determined by appended claims and their equivalents.

What is claimed is:

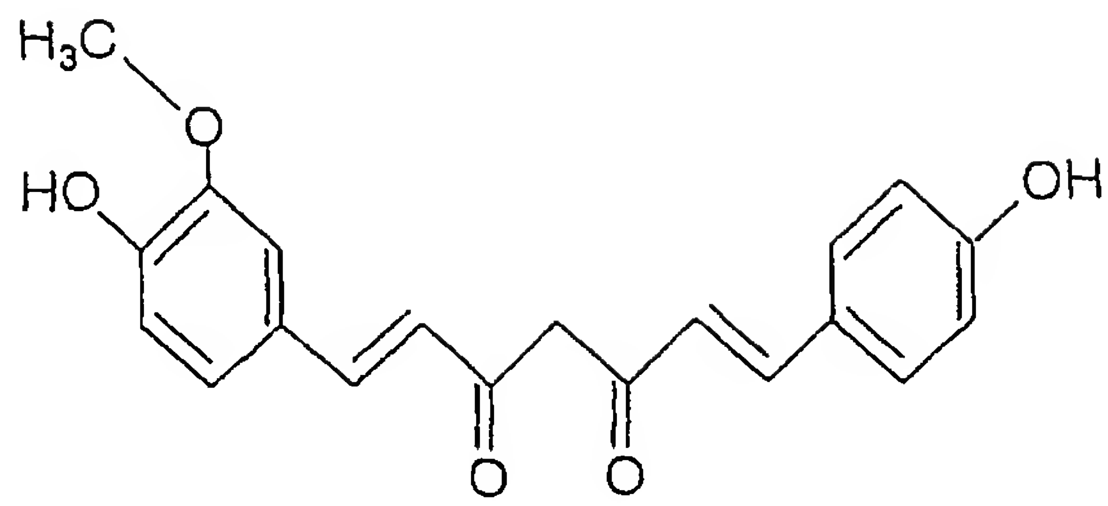
1. A curcumin derivative represented by the following formula I:



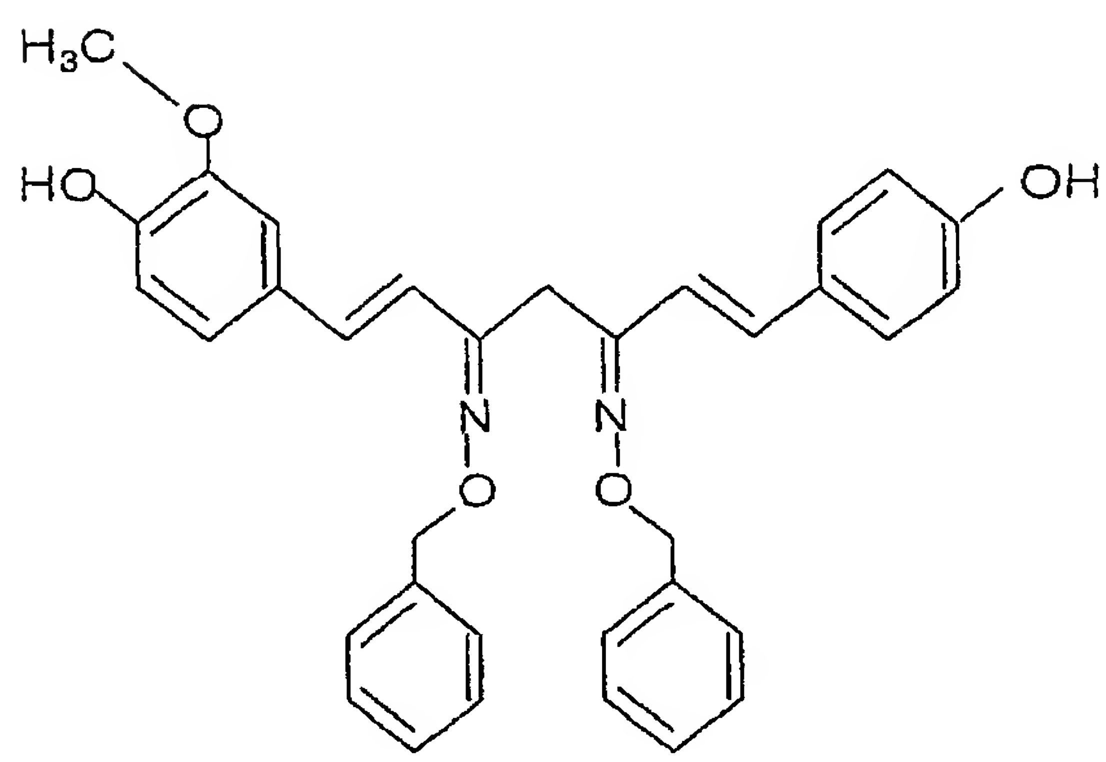
(I)

wherein R_1 represents H or lower alkyl group of 1-4 carbon atoms, R_2 represents H or lower alkoxy group of 1-4 carbon atoms, R_3 represents H or lower alkoxy group of 1-4 carbon atoms, R_4 represents H or lower alkyl group of 1-4 carbon atoms and both of R_5 and R_6 represent nitrogen or oxygen atoms; in which when both of R_5 and R_6 are nitrogen atoms, each of R_5 and R_6 is substituted with $-OR_7$ and R_7 is H, alkyl, cycloalkyl, aryl, alkaryl or aralkyl, or R_5 and R_6 form a ring structure with a hydrazine group and R_5 and R_6 are unsubstituted or independently substituted with alkyl, cycloalkyl, aryl, alkaryl or aralkyl; and in which when R_1 is H, R_2 is not methoxy, R_3 is not H or methoxy group, R_4 not H and both of R_5 and R_6 not oxygen.

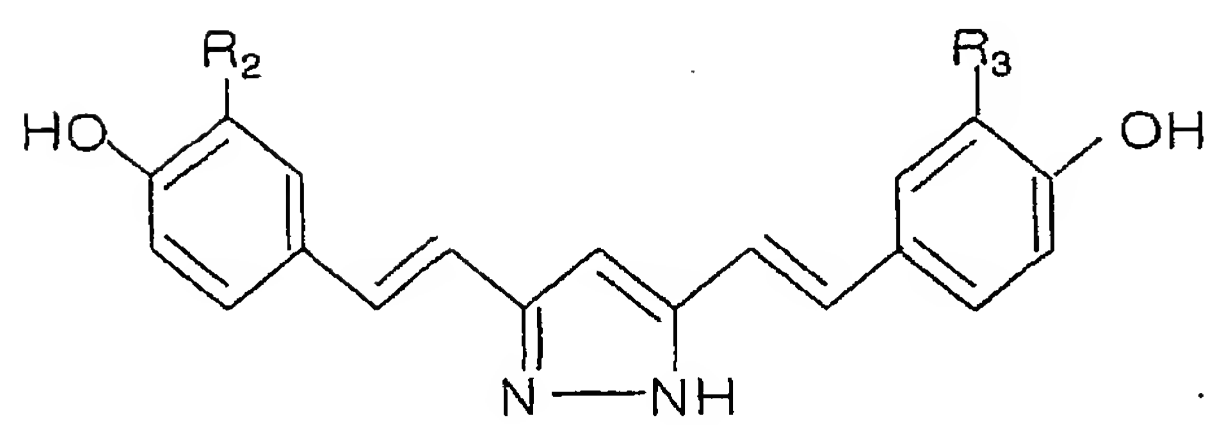
2. The curcumin derivative according to claim 1, wherein the derivative is represented by any one of the following formulae II, III, IV, V or VI:



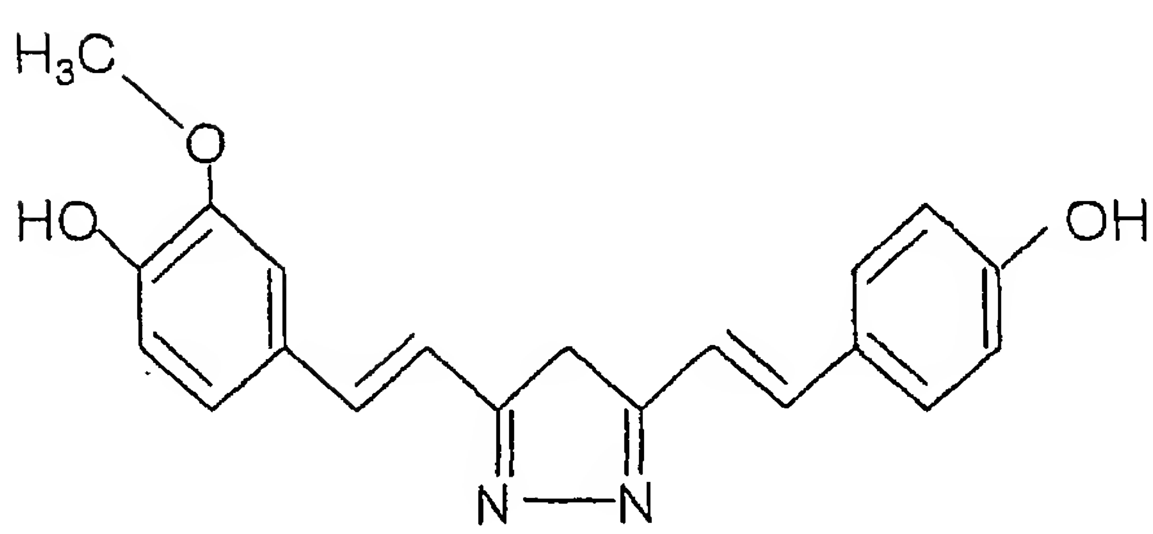
(II)



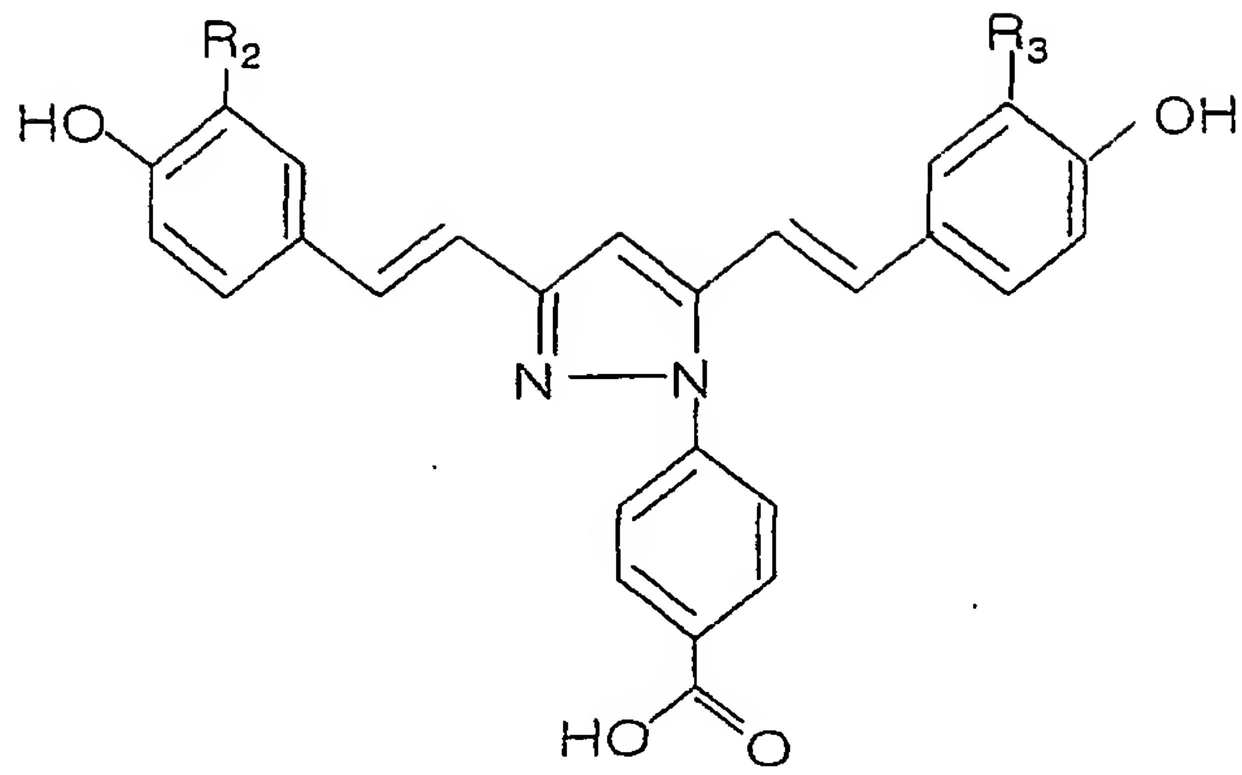
(III)



(IV)



(V)



(VI)

wherein R_2 and R_3 are the same as those in formula I.

3. The curcumin derivative according to claim 1, wherein R_2 and R_3 in formula independently represent H or methoxy group.

4. The curcumin derivative according to claim 2, wherein R_2 and R_3 in formula independently represent H or methoxy group.

5. A pharmaceutical composition for treating or preventing a disease associated with unregulated angiogenesis, which comprises: (a) a pharmaceutically effective amount of the curcumin derivative according to any one of claims 1-4; and (b) a pharmaceutically acceptable carrier.

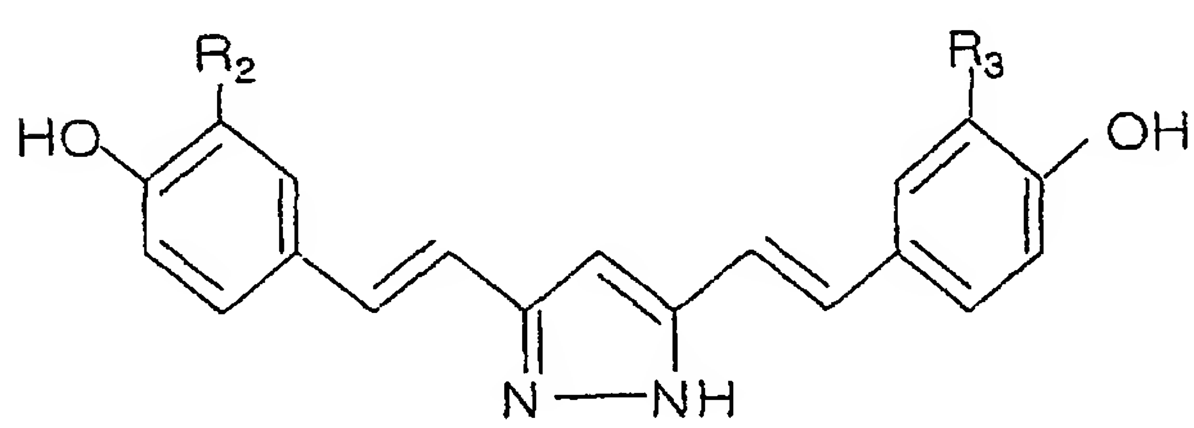
6. The pharmaceutical composition according to claim 5, wherein the disease associated with unregulated

angiogenesis is selected from the group consisting of rheumatoid arthritis, diabetic retinopathy, cancer, hemangioma and psoriasis.

7. The pharmaceutical composition according to claim 6, wherein the disease associated with unregulated angiogenesis is cancer.

8. The pharmaceutical composition according to claim 5, wherein the curcumin derivative is a hydrazinocurcumin.

9. The pharmaceutical composition according to claim 8, wherein the hydrazinocurcumin is represented by the following formula IV:



(IV)

wherein R_2 and R_3 are the same as those in formula I.

10. The pharmaceutical composition according to claim 9, wherein R_2 and R_3 in formula independently represent H or methoxy group.

11. A method for extracting from *Curcuma aromatica* curcumin and derivatives thereof, which comprises

contacting *Curcuma aromatica* with 70-98% methanol solution for 2-5 hours under heat treatment at 55-65℃.

12. The method according to claim 11, wherein the method comprises contacting *Curcuma aromatica* with about 95% methanol solution for about 3 hours under heat treatment at about 60℃.

13. The method according to claim 11, wherein the derivative of curcumin is demetoxycurcumin or bisdemetoxycurcumin.

Fig. 1

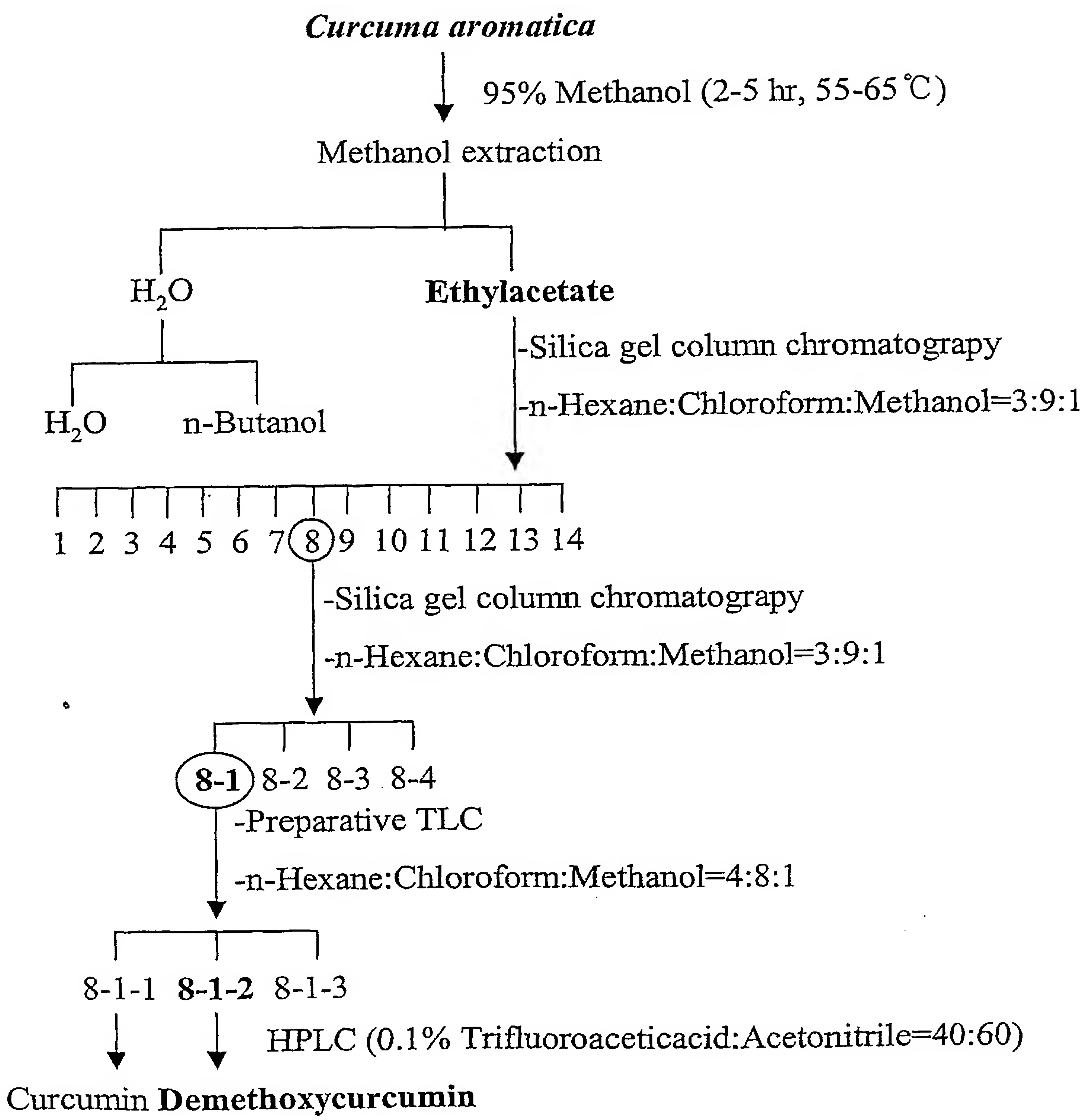
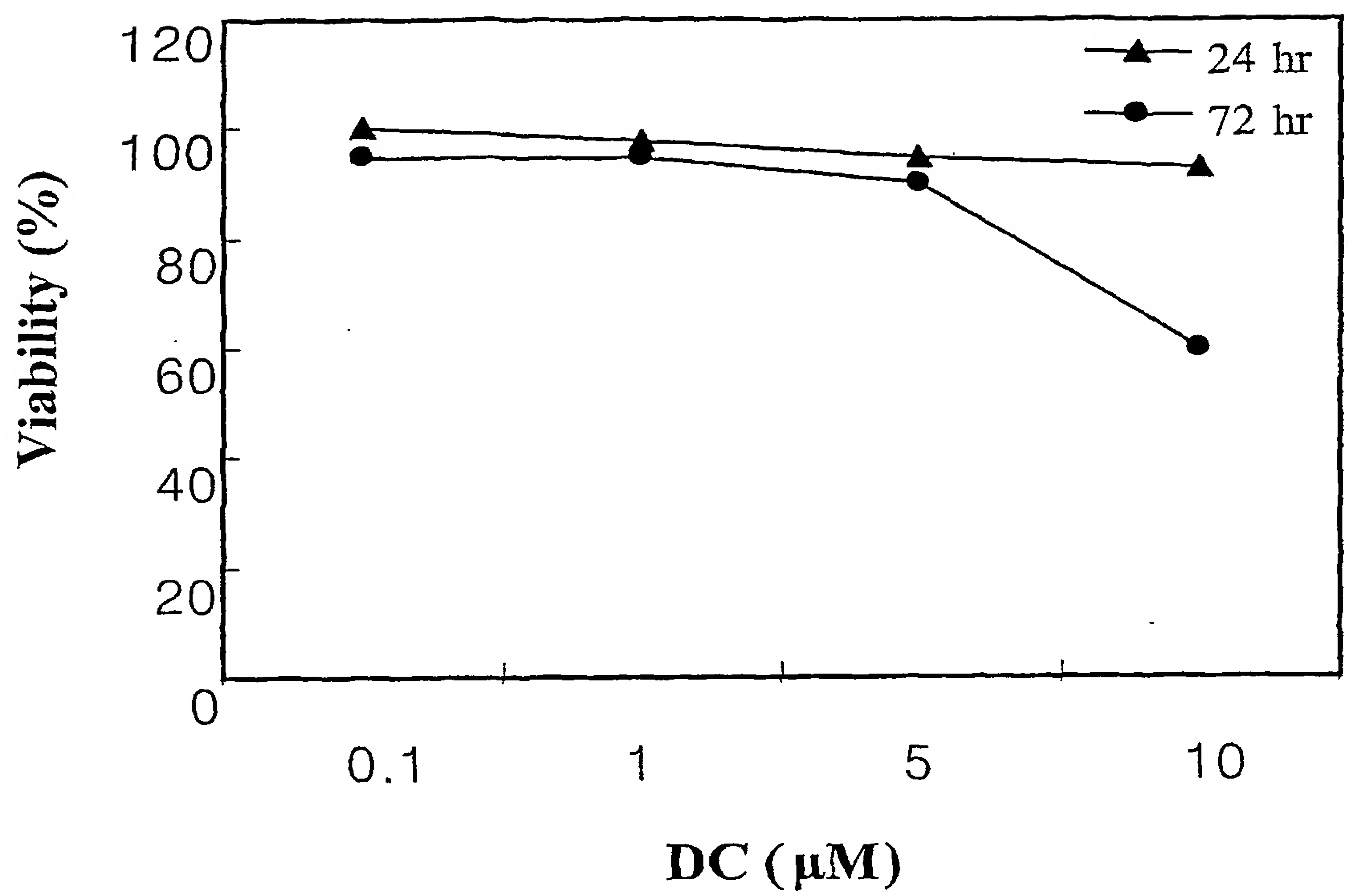

Curcuma aromatica
95% Methanol (2-5 hr, 55-65℃)
Methanol extraction
H2O
Ethylacetate
-Silica gel column chromatograpy
-n-Hexane:Chloroform:Methanol=3:9:1
H2O
n-Butanol
1 2 3 4 5 6 7 8 9 10 11 12 13 14
-Silica gel column chromatograpy
-n-Hexane:Chloroform:Methanol=3:9:1
8-1 8-2 8-3 8-4
-Preparative TLC
-n-Hexane:Chloroform:Methanol=4:8:1
8-1-1 8-1-2 8-1-3
HPLC (0.1% Trifluoroaceticacid:Acetonitrile=40:60)
Curcumin Demethoxycurcumin

Fig. 2

A **HUVECs**



B **BAECs**

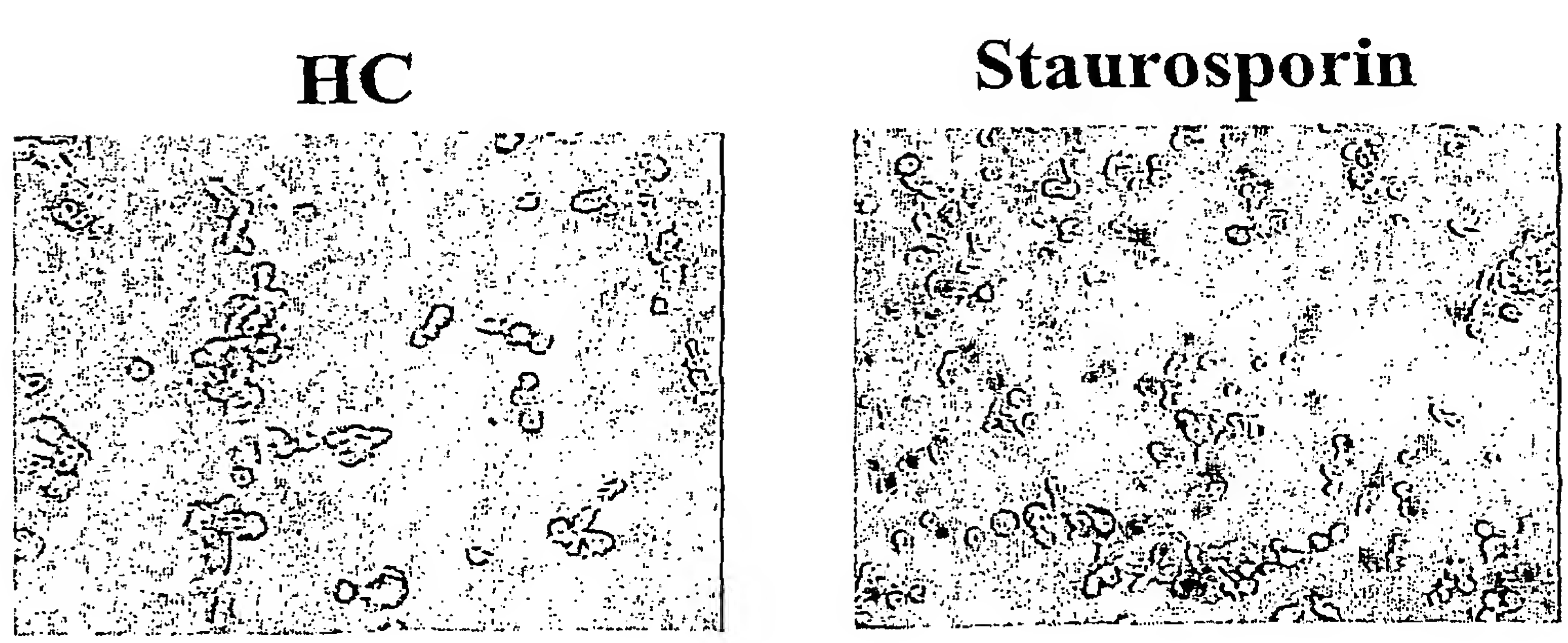


Fig. 3

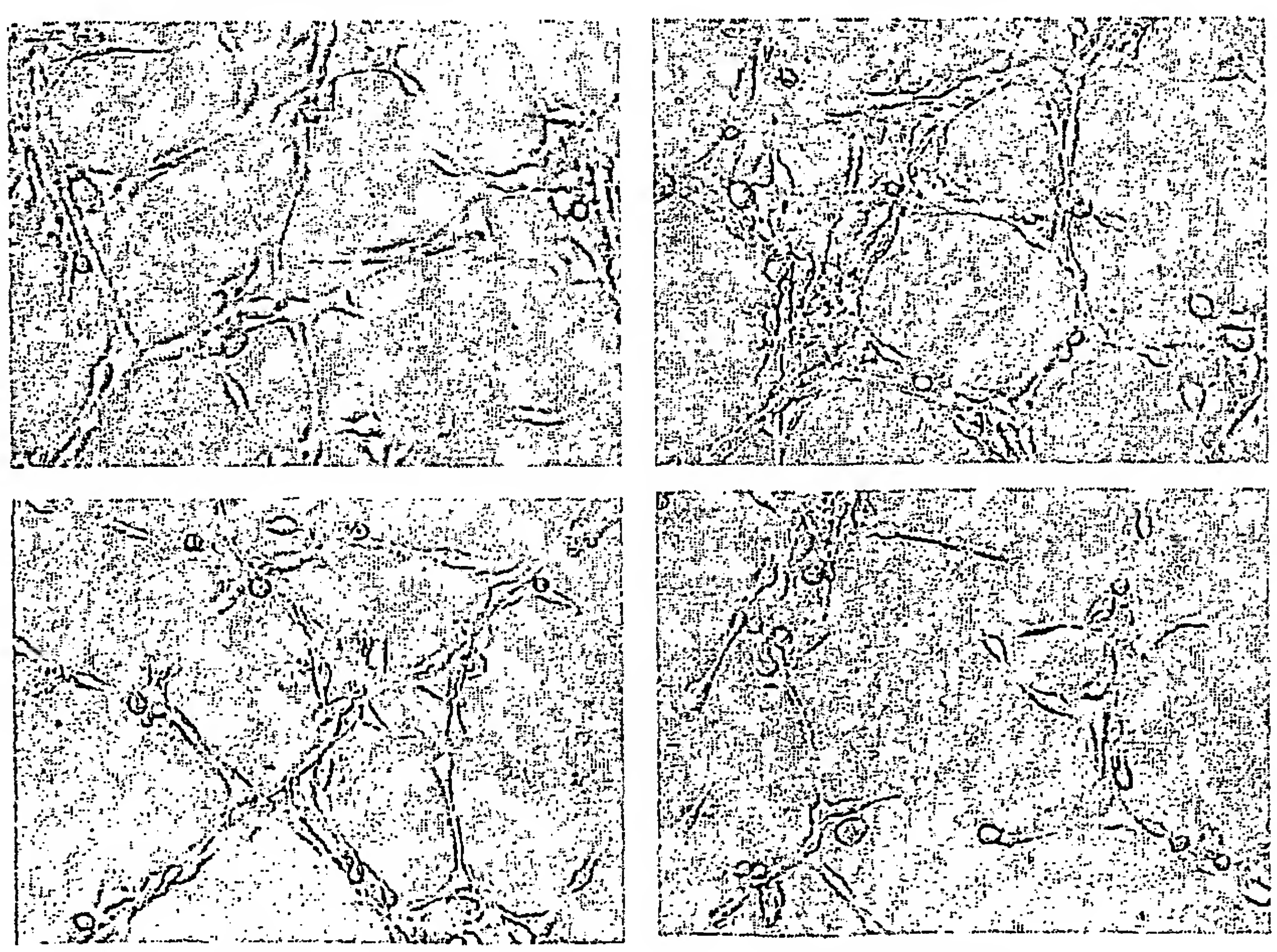
A HUVECs

Control bFGF bFGF+5μM DC

Control bFGF

B

BAECs



bFGF+ HC(100nM) bFGF+ HC(300nM)

Fig. 4

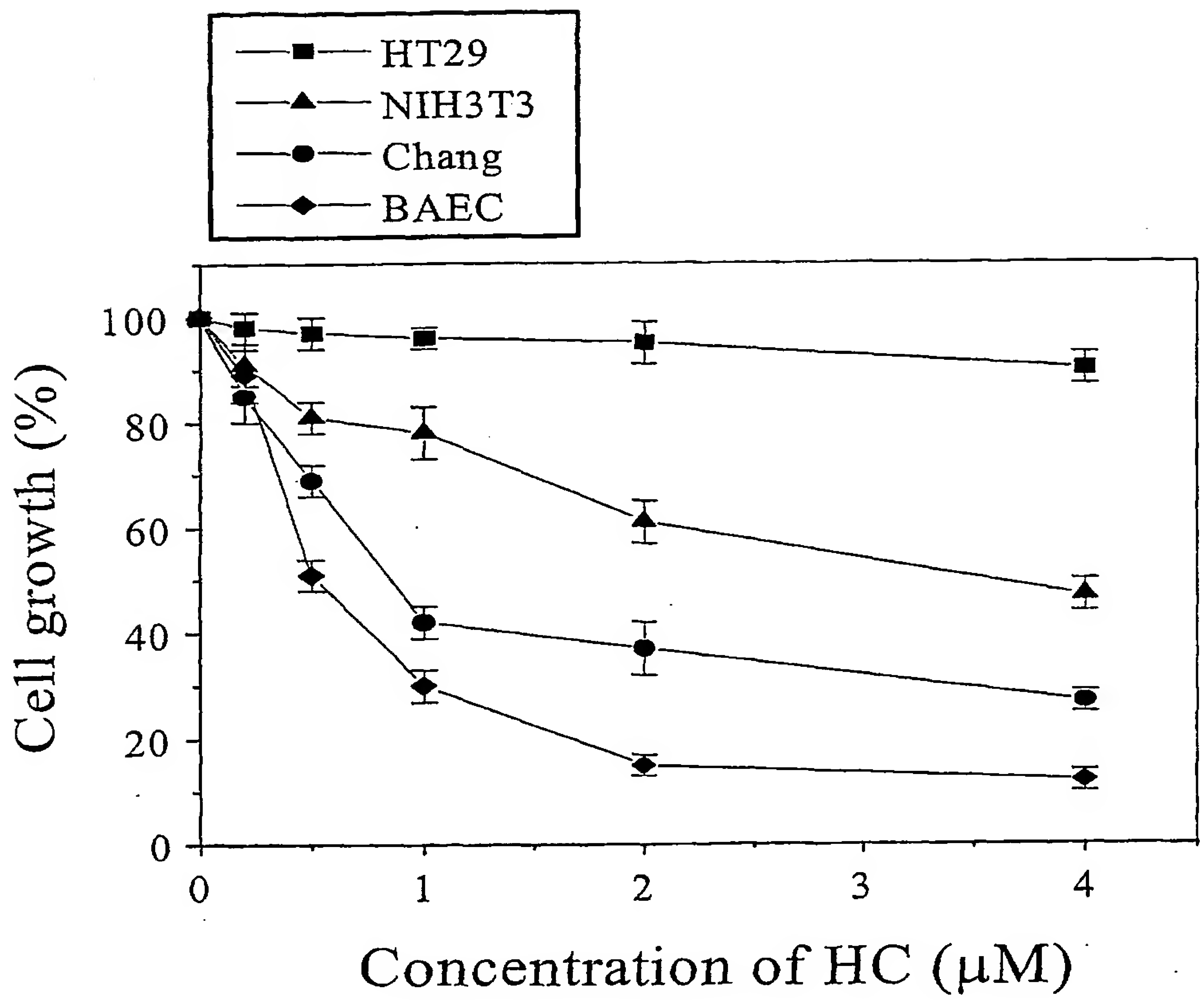
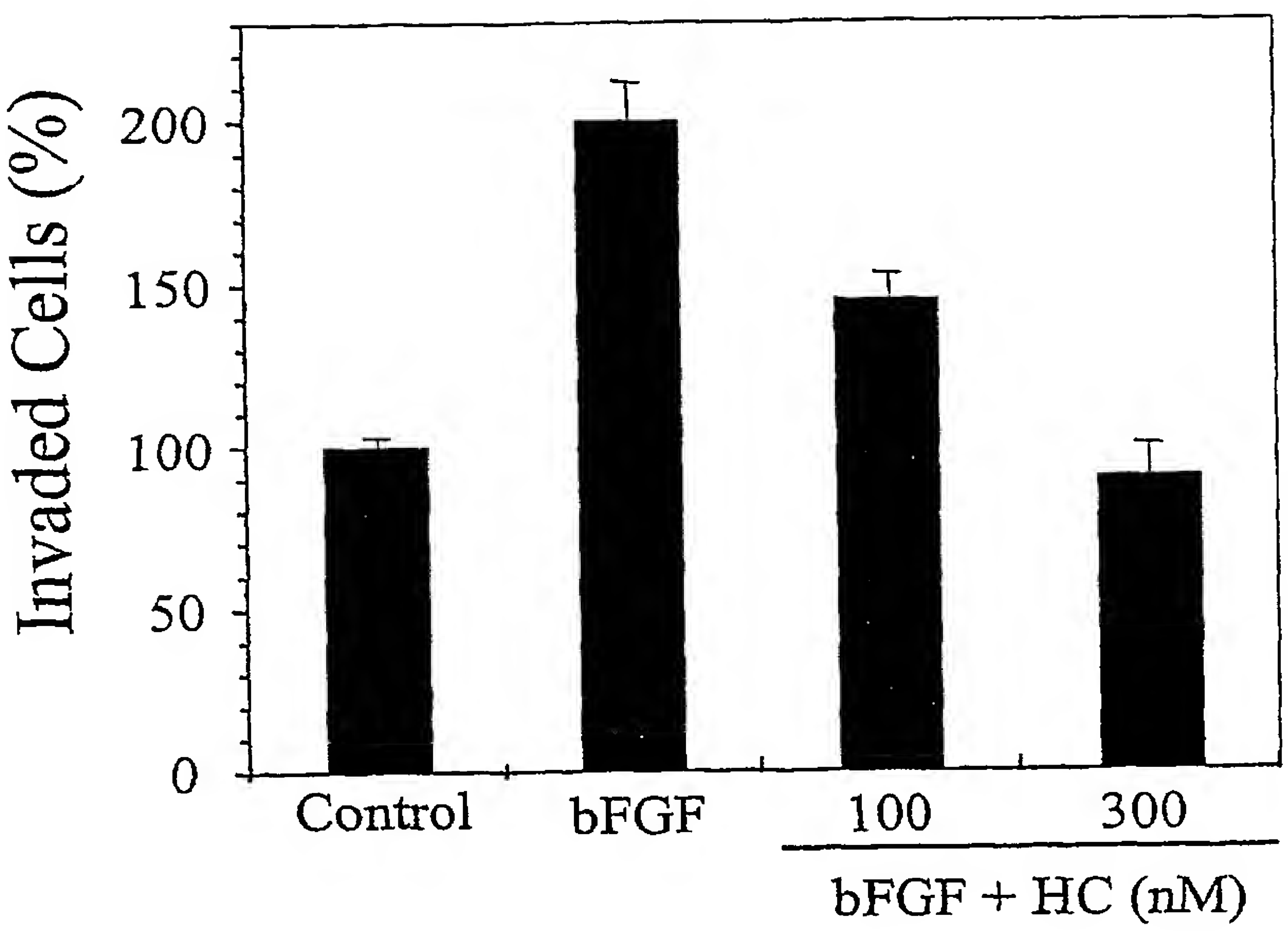
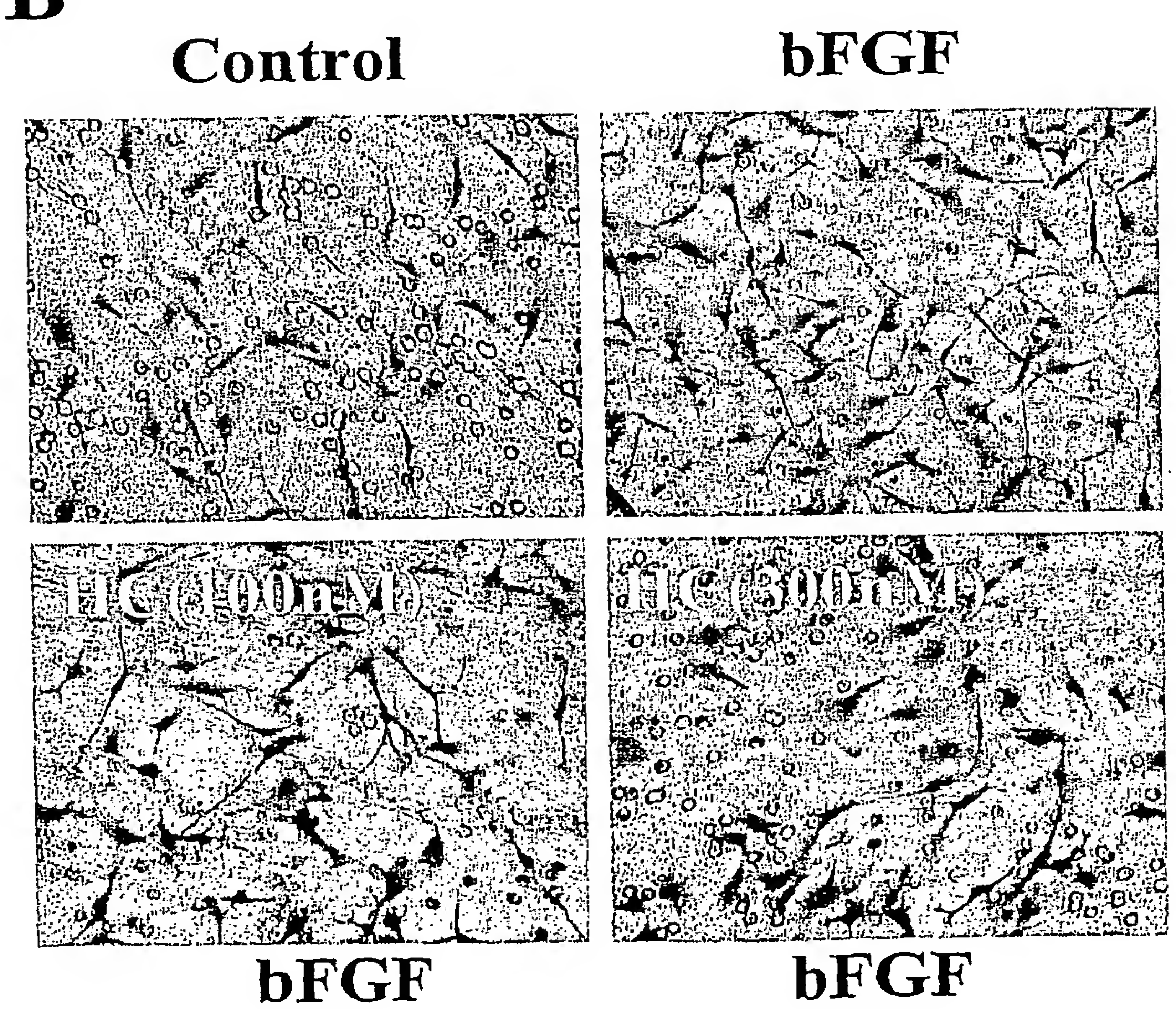

HT29
NIH3T3
Chang
BAEC
Cell growth (%)
100
80
60
40
20
0
0
1
2
3
4
Concentration of HC (μM)

Fig. 5

A


Invaded Cells (%)
200
150
100
50
0
Control
bFGF
100
300
bFGF + HC (nM)

B


Control
bFGF
HC (100nM)
HC (300nM)
bFGF
bFGF

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: NOVEL CURCUMIN DERIVATIVES

(57) Abstract: The present invention relates to a novel curcumin derivative and a pharmaceutical composition, in particular to a novel curcumin derivative with anti-angiogenic activity and a pharmaceutical composition for treating or preventing a disease associated with unregulated angiogenesis.

INTERNATIONAL SEARCH REPORT

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CLASSIFICATION OF SUBJECT MATTER

IPC[7]: C07C 49/223, 49/248, 251/36, C07D 231/12, A61JK 31/121, 31/15, 31/415

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: C07C, C07D, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REGISTRY, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	US 2003203933 A1 (Lee, Kuo-Hsiung; Ishida, Junko; Ohtsu, Hironari; Wang, Hui-Kang; Itokawa, Hideji; Chang, Chawnshang; Shih, Charles C. Y.; University of North Carolina at Chapel Hill, USA) 30 October 2003 (30.10.2003) *compounds 2-9 (fig. 1), claims 1-7, 17-20.*	1-10
X	WO 2001/000201 A1 (Emory University, USA) 4 January 2001 (04.01.2001) *claims 4, 9,10, 12, 13.*	2, 5-7
X	EP 0245825 A1 (Warner-Lambert Co., USA) 19 November 1987 (19.11.1987) *example 1, claim 1.*	1-4

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 May 2004 (13.05.2004)	22 June 2004 (22.06.2004)
Name and mailing adress of the ISA/AT Austrian Patent Office Dresdner Straße 87, A-1200 Vienna Facsimile No. 1/53424/535	Authorized officer MÜLLER-HIEL R. Telephone No. 1/53424/434

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chowdhury, Hemanta; Walia, Suresh; Saxena, Vinod S. "Isolation, characterization and insect growth inhibitory activity of major turmeric constituents and their derivatives against Schistocerca gregaria (Forsk) and Dysdercus koenigii (Walk)" Pest Management Science (2000), 56(12), 1086-1092 (abstract). Chemical Abstracts [online] Copyright 2004 American Chemical Society [retrieved on 11 May 2004 (11.05.2004)]. Retrieved from STN International, Karlsruhe. Chem. Abstr. No. 2000:872086. *abstract, compounds RN 316148-96-6, 316148-97-7, 316148-98-8, Demthoxycurcumine, Bisdemethoxycurcumine.*	1-4
X	WO 1997/016403 A1 (Geneprint, Inc., USA) 9 May 1997 (09.05.1997) *example 45.*	1
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Remarks:

Claims 1 and 2 are unclear, because of the disclaimer in claim 1. Disclaimed are compounds of formula (I), wherein
"when R1 is H,
R2 is not methoxy, R3 is not H or methoxy group, R4 is not H, and both of R5 and R6 are not oxygen"
This denotes the disclaiming of curcumine (R1, R4 are H; R2, R3 are methoxy, R5, R6 are O) and demethoxycurcumine (R1, R3, R4 are H; R2 is methoxy; R5, R6 are O)
Therefore, compound (II) claimed in dependent claim 2 is one of the compounds disclaimed in in claim 1.

Claim 4 is unclear, because reference is made to an unnumbered formula according to claim 2, whereas claim 2 covers several formulas.

Claims 11 and 12 are unclear, because no structural definitions of the curcumin derivatives are given.

The method of preparation curcumin derivatives according to claims 11 and 12 is not sufficiently supported by the description, because in the description the method does not apply to other curcumin derivatives than demthoxycurcumine and bisdemethoxycurcumine.

It is noted, that unity of claims 11-13 (method of preparation curcumin derivatives) and claim 1 (curcumin derivatives) is given only, if the method applies to curcumin derivatives which are subject of claim 1.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
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